Direct Amino Acid Uptake by Plants

related to Grassland Diversity

-

methodological and ecological Investigations

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by Leopold Sauheitl

>>In nature, one plus one
is not necessarily always two
–
and even the old wise men
in the Israelite desert know this.<<

Prof. Dr. Daniel Hillel
EUROSOIL congress 2008

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Abbreviations

10Me16:0	10-methylhexadecanoic acid
10Me17:0	10-methylheptadecanoic acid
10Me18:0	10-methyloctadecanoic acid
12C	stable carbon isotope with atom mass 12
13C	stable carbon isotope with atom mass 13
14:0	tetradecanoic acid
14C	radioactive carbon isotope with atom mass 14
14N	stable nitrogen isotope with atom mass 14
15:0	pentadecanoic acid
15N	stable nitrogen isotope with atom mass 15
16:0	hexadecanoic acid
16:1w5c	cis-11-hexadecenoic acid
16:1w7c	cis-9-hexadecenoic acid
17:0	heptadecanoic acid
18:0	octadecanoic acid
18:1w7c	cis-octadecenoic acid
18:1w9c	cis-9-octadecenoic acid
18:2w6,9	cis,cis-9,12-octadecadienoic acid
20:4w6	cis,cis,cis,cis-5,8,11,14-eicosatetraenoic acid
a15:0	12-methyltetradecanoic acid
a17:0	14-methylhexadecanoic acid
AA	amino acid
ACC	trans-4-(aminomethyl) cyclohexane carboxylic acid
aIle	allo-isoleucine
Ala	alanine
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
Asn	asparagine
Asp	aspartic acid
Bq	Becquerel
CFE	Chloroform Fumigation Extraction
CSI	Compound Specific Isotope

cy17:0	cis-9,10-methylenhexadecanoic acid
cy19:0	cis-9,10-methylenoctadecanoic acid
EA	Elemtal Analyser
F	F-value in ANOVA
G-	Gram negative bacteria
G+	Gram positive bacteria
GC	Gas Chromatograph
GLM	General Linear Model
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
i15:0	13-methyltetradecanoic acid
i16:0	14-methylpentadecanoic acid
i17:0	15-methylhexadecanoic acid
Ile	isoleucien
IRMS	Isotope Ratio Mass Spectrometer
IS1	internal standard 1
IS2	internal standard 2
Leu	leucine
LMWOS	low molecular weight organic substance
Lys	lysine
M	molar [mol*L-1]
Met	metionine
Nmin	mineral N
Nor	norvaline
Orn	ornitin
p	Probability in statistics
PE	polyethylen
PEP	phosphoenolpyruvat carboxylase
Phe	phenylalanine
PLFA	Phospho Lipid Fatty Acid
Pro	proline
R	atom ratio of heavy isotope : light isotope

Ser	serine
SR	species richness
TCA	tri carbonic acid cycle
Val	valine
VAM	Visicular Arbuscular Mycorrhiza

Summary

Uptake of intact amino acids by plants has been identified as an alternative nitrogen (N) source for plants in a number of ecosystems and soil types. Up to now it is assumed that this uptake strategy is of particular relevance in ecosystems with low mineral N contents due to insignificant microbial activity (low soil temperatures) or due to poorly developed soils. However, it has also been discussed that amino acid uptake might enable plants to lower intracspecific competition for mineral nitrogen and shortcut the microbial mineralization of organic N in systems were competition is exceptionally high. The positive effect of plant diversity on plant productivity is known to induce these conditions of intense N competition and thus amino acid uptake might explain how plant communities manage to be more productive with increasing diversity. However, the ecological importance of organic N uptake has as well been questioned, due to the high competitive power of microbes in soils of the temperate zone and especially due to a number of flaws in the commonly used method to proof and quantify direct amino acid uptake. In this, dual labelled (^{13}C and ^{15}N) amino acids are injected into the soil and direct tracer uptake is quantified via bulk isotope measurement of ^{13}C and ^{15}N enrichment in plant tissues, which recently has been challenged to exclusively reflect direct amino acid uptake.

The first objective therefore was to identify and reduce methodological influences on the direct amino acid uptake by plants. In this context the effect of changed amino acid concentrations on amino acid uptake was investigated by application of different tracer amounts. Next, the accuracy and precision of commonly used bulk isotope measurements were compared to compound specific measurements with respect to the determination of direct amino acid uptake.

It could be shown that the use of high tracer amounts accompanied by high mineral N release led to a decrease of direct amino acid uptake via plant internal down regulation of amino acid transporters. This corroborates the importance of minimizing tracer amounts and suggests that plants can actively increase amino acid uptake when N availability in soil is low. Bulk measurements turned out to overestimate direct amino acid uptake by a factor of up to six, as they were not able to separate uptake of intact tracer molecules from uptake of tracer fragments or inorganic carbon. At the same time compound specific isotope measurements proofed to be an accurate and precise tool to demonstrate and quantify uptake of intact amino acids.

Using these optimized methods, the importance of direct amino acid uptake for the N-nutrition of plants with respect to changing plant diversity was investigated. The uptake of amino acids and mineral N by plants as well as the competition between plants and microbes for amino acid-N was investigated in grassland communities with 1 to 16 grassland species. To draw a complete picture of the interactions between plant diversity and belowground N dynamics as driven by the microbial community, the effect of plant diversity on composition and activity of the microbial community was additionally investigated. Microbes were superior competitors for amino acid derived nitrogen, irrespective of plant diversity and took up 54 % of the applied amino acid N in average within 24 h. In contrast, plants only incorporated 2.68 % of the applied N and were thus less effective by a factor of 20 in short term N acquisition than microbes. In addition, plant mineral N uptake decreased with increasing plant diversity while the uptake of intact amino acids increased. Therewith the contribution of amino acid uptake to the overall plant N nutrition increased from 1.47 to 7.04 % in which amino acid uptake was mainly controlled by plant parameters such as plant N-concentration shoot biomass and rooting density while mineral N uptake was controlled by microbial competition. In detail amino acid uptake increased with decreasing plant N concentration while mineral N uptake decreased with increasing microbial abundance and microbial N uptake. Therefore, the boosted importance of amino acid uptake for plant N nutrition has to be seen as a reaction on increased N competition with increasing plant diversity.

Additionally, plant diversity stimulated microbial diversity which was most likely due to the bottom up effect of increased root exudation and litter input caused by increasing N competition and plant productivity, respectively. While the microbial community in general was dominated by bacteria (54.7 %) the abundance of litter and soil organic matter decomposing gram positive bacteria and fungi as well as protozoan abundance increased with increasing plant diversity. Protozoa are known to stimulate turnover of bacteria which was indicated by higher tracer incorporation by this microbial group and an overall increase of deaminase activity with increasing plant diversity. As a higher microbial turnover is associated with an increased loss of microbial N to plants, we have to expect higher N availability for plants in the long term.

The positive feedback of a plant-induced higher microbial turnover rate on N availability in soil together with the increased use of amino acids as alternative N source might therefore be and important model to explain the positive effect of plant diversity on plant productivity.

Zusammenfassung

Die pflanzliche Aufnahme intakter Aminosäuren als alternative Stickstoffquelle wurde in einer Reihe von Ökosystemen und Bodentypen nachgewiesen. Dabei wird davon ausgegangen, dass Pflanzen in Böden mit niedrigen mineralischen N (N_{min}) Gehalten oder starker N-Konkurrenz durch die Aufnahme von Aminosäuren die mikrobielle N-Mineralisation umgehen und die intraspezifische Konkurrenz um N_{min} vermindern können. Daneben ist bekannt, dass der positive Einfluss pflanzlicher Diversität auf die Pflanzenproduktivität zu verstärkter N-Konkurrenz führt. Daher könnte die direkte Aminosäureaufnahme möglicherweise erklären, wie Pflanzen bei höherer Diversität produktiver sein können. Allerdings wurde die tatsächliche ökologische Bedeutung der Aminosäureaufnahme immer wieder angezweifelt, was mit der großen mikrobiellen Konkurrenzstärke in Böden der gemäßigten Breiten sowie vor allem mit den Schwächen der standardmäßig verwendeten Nachweis- und Quantifizierungsmethode begründet wurde. In dieser Methode wird die bulk ^{13}C und ^{15}N Anreicherung in Pflanzenmaterial nach der Applikation von doppelt markierten (^{13}C und ^{15}N) Aminosäuren zur Berechnung der direkten Aminosäureaufnahme verwendet. Kürzlich wurde aber angezweifelt, dass dies tatsächlich nur die Aufnahme intakter Tracermoleküle reflektiert.

Die erste Zielsetzung war es daher die methodischen Einflüsse auf die direkte Aminosäureaufnahme zu identifizieren und zu reduzieren. Dazu wurde der Effekt unterschiedlicher Aminosäurekonzentrationen im Boden auf die Aminosäureaufnahme durch die Applikation verschiedener Tracermengen untersucht. Außerdem wurde Richtigkeit und Präzision bisher verwendeter Bulk Isotopenmessungen bei der Quantifizierung der Aminosäureaufnahme mit substanzspezifischen Messungen (CSI) verglichen.

Die Verwendung höherer Tracermengen führte dabei zu einer verstärkten N-Mineralisation und dadurch zu einer Herunterregulierung der pflanzlichen Aminosäuretransporter, gefolgt von einer geringeren Aminosäureaufnahme. Dies unterstreicht einerseits die Notwendigkeit einer Minimierung von Tracermengen und zeigt andererseits, dass Pflanzen bei niedriger N-Verfügbarkeit ihre Aminosäureaufnahme aktiv erhöhen können. Außerdem zeigte es sich, dass Bulk Messungen nicht geeignet sind um zwischen der Aufnahme intakter Tracermoleküle und der Aufnahme tracerbürtiger C-Bruchstücke oder anorganischem C zu differenzieren, wodurch die Aminosäureaufnahme um das Sechsfache überschätzt wird. Gleichzeitig erwiesen sich CSI Messungen als geeignetes Mittel um die Aminosäureaufnahme richtig und präzise zu quantifizieren.

Unter Verwendung dieser optimierten Methoden wurde die Bedeutung der Aminosäureaufnahme für die pflanzliche N-Ernährung im Hinblick auf eine veränderte pflanzliche Diversität untersucht. Sowohl die pflanzliche Aminosäure- und N_{min}-Aufnahme als auch die Konkurrenz um Aminosäure-N zwischen Pflanzen und Mikroben wurde in Grasslandsystemen mit 1 bis 16 Pflanzenarten untersucht. Um die Interaktionen zwischen pflanzlicher Diversität und der N-Dynamik im Boden in ihrer Gänze erfassen zu können, wurde außerdem der Diversitätseffekt auf Zusammensetzung und Aktivität der mikrobiellen Gemeinschaft untersucht.

Unabhängig von der pflanzlichen Diversität waren Mikroben in der Aneignung tracerbürtigen Stickstoffs mit einer mittleren Aufnahme von 54 % des applizierten N den Pflanzen überlegen, die innerhalb derselben Zeit (24 h) nur 2.68 % aufnahmen. Zusätzlich verringerte sich die pflanzliche N_{min}-Aufnahme mit höher werdender Diversität, während die Aminosäureaufnahme zunahm. Damit erhöhte sich der Beitrag der Aminosäureaufnahme zur gesamten N-Aufnahme von 1.47 % auf 7.04 %. Im Einzelnen stieg die Aminosäureaufnahme bei niedrigeren pflanzlichen N-Konzentrationen an, während die N_{min}-Aufnahme mit größer werdender mikrobieller Abundanz und N-Aufnahme abnahm. Damit kann die verstärkte Aminosäureaufnahme als Reaktion auf eine verstärkte Konkurrenz um N bei größerer pflanzlicher Diversität interpretiert werden.

Gleichzeitig wurde ein positiver Einfluss der pflanzlichen auf die mikrobielle Diversität festgestellt. Dies wurde vermutlich durch den „Bottom-up“ Effekt von Wurzelexudation und Streueintrag verursacht, die beide aufgrund von verstärkter N-Konkurrenz und pflanzlicher Produktivität mit erhöhter Diversität zunahmen. Während die mikrobielle Gemeinschaft grundsätzlich bakteriell dominiert war (54 %), erhöhte sich die Abundanz von Streu und organische Bodensubstanz abbauenden Gram positiven Bakterien und Pilzen sowie von Protozoen mit ansteigender pflanzlicher Diversität. Es ist bekannt, dass Protozoen den gesamten bakteriellen Umsatz ankurbeln, was sich in verstärktem bakteriellen Tracerumsatz und erhöhter Deaminaseaktivität bei höherer Diversität manifestierte. Da höherer bakterieller Umsatz mit einem verstärkten Verlust von mikrobiell gebundenen N an Pflanzen einhergeht, muss davon ausgegangen werden, dass dies langfristig zu einer erhöhten N-Verfügbarkeit für Pflanzen führt.

Die positive Rückkopplung des pflanzlich induzierten höheren mikrobiellen Umsatzes auf die N-Verfügbarkeit im Boden zusammen mit der verstärkten Nutzung von Aminosäuren als alternative N-Quelle könnte damit ein wichtiges Modell zur Erklärung des positiven Produktivitätseffektes erhöhter pflanzlicher Diversität sein.

I) Extended Summary

1. Introduction

1.1 Preface

The doctrine that nitrate and ammonium are the exclusive nitrogen sources for plants has been disproved as early as 1946, when Virtanen et al. (1946) showed that peas and clover are capable of taking up intact aspartic acid from sterile hydroponic solutions via their root system. However, for the next five decades, the ability of plants to use organic nitrogen (ON) has been widely neglected in natural terrestrial ecosystems were plants were thought to be completely outcompeted for ON uptake by soil microbes (Black, 1993). While the interest in the use of ON started to increase again in the 80's of the last century (Stribley and Read, 1980; Read and Bajwa, 1985), it was still difficult to proof and quantify plant uptake of intact amino acids in the field. With the increasing use of stable isotope tracers it became easier to investigate nutrient fluxes in soil and detect and quantify nitrogen sources used by plants, even in the field. By means of this technique it was possible to proof amino acid uptake for a number of different plants, including grasses and shrubs, in a wide range of ecosystems and climates. These investigations comprised Alaskan tussock tundra (Kielland, 1994), boreal coniferous forest (Näsholm et al., 1998), heathland (Stribley and Read, 1980), subtropical rainforest (Schmidt and Stewart, 1999) and even agricultural land (Jones and Darrah, 1994; Yamagata and Ae, 1996; Näsholm et al., 2001a). Along with the investigation of amino acid uptake in the field, the mechanistic aspects of the uptake process were elucidated in a number of studies (Blackman and McDaniel, 1980; McDaniel et al., 1982; Wyse and Komor, 1984; Datko and Mudd, 1985; Schneegurt and McDaniel, 1986), together with the identification of various amino acid transporters in plant roots (Fischer et al., 1998; Liu and Bush, 2006).

1.2 Possible ecological impact of amino acid uptake

Any uptake of organic N by plants has to be seen as an alternative N source, which in addition to nitrate and ammonium uptake could help to cover the N demand of plants. For this reason, plant amino acid uptake was first investigated in soils that were dominated by free amino acids, e.g. in arctic tundra (Kielland, 1990), boreal forests and alpine ecosystems, and where microbial N mineralization was insufficient to account for

the annual N uptake by plants (Rehder and Schäfer, 1978; Kielland, 1990; Kielland, 1994; Fisk and Schmidt, 1995; Kaye and Hart, 1997). Under these conditions, amino acid uptake accounted for 60 % of the total N uptake of a non-mycorrhized arctic sedge (Chapin et al., 1993) and for 10 – 82 % of a set of different arctic plants (Kielland, 1994). These results do not only show the high ecological impact of amino acid uptake for plant N nutrition under these harsh environmental conditions but also give evidence for the high variability of amino acid uptake between plant species which suggests niching of plant species with respect to the used N source (Kielland, 1994).

However, for ecosystems of the temperate zones, the ecological impact of amino acids still is doubted as due to higher soil temperatures microbial activity is high and microbes have been shown to be superior competitors for amino acids (Jones, 1999; Jones and Hodge, 1999; Jones et al., 2005b). Indeed, the contribution of amino acid N to the total plant N uptake in these ecosystems is lower than for arctic and subarctic ones, e.g. 3.8 – 53.6 % for five temperate grassland species (Weigelt et al., 2005), 19 – 23 % for four agricultural plants (Näsholm et al., 2000). But even when the plant N nutrition was dominated by mineral N uptake, differences between plant species in amino acid uptake were marked (Weigelt et al., 2005) and plant species were shown to adapt their N nutrition patterns to N availability in soil (Weigelt et al., 2003). According to these data, uptake of intact amino acids can contribute significantly to plant N nutrition even in temperate climates and facilitate niching of plants as a consequence of changing N availabilities in soil.

1.3 Relevance of amino acid uptake in plant diversity experiments

It is of accepted knowledge that a man-made change of plant diversity compromises the goods and services these ecosystems provide for mankind, e.g. the storage of carbon, the retention of nutrients in soil and the control of plant pests (Hooper et al., 2005). Thus, ecological research of the last two decades has focused on the question which mechanisms form the basis of these biodiversity effects. Increased plant productivity, as frequently found in plant diversity experiments (Tilman et al., 1996; Hector et al., 1999; Loreau et al., 2001; Hooper et al., 2005; Spehn et al., 2005; van Ruijven and Berendse, 2005; Marquard et al., 2009), might be the main factor mediating increased carbon storage (Gleixner et al., 2005; Steinbeiss et al., 2008) and nutrient retention (Naeem et al., 1994; Naeem et al., 1996). Thus, one central point of interest is the question how plants manage to be more productive with increasing diversity. There is strong evidence that an increased productivity is facilitated by an increased use efficiency of

growth limiting resources (Hooper et al., 2005) by source partitioning between plants, i.e. the use of the same resource at different time or at different points in space or uptake of the same nutrient but in different chemical forms. This can be caused by the combination of plant species of different ecophysiological potential (complementarity) or by species plasticity with respect to resource use (niching). As a consequence, the overall use of plant-available resources is suggested to increase (Trenbath, 1974; Harper, 1977; Ewel, 1986; Vandermeer, 1989) and thus productivity ought to increase with increasing diversity. One of the main growth limiting resources in terrestrial ecosystems is nitrogen (Vitousek and Howarth, 1991). This is supported by the positive effect of legumes on the plant productivity-diversity relationship, in which a number of studies have shown that increased plant productivity with increasing diversity is stimulated under the presence of legumes (Hooper and Vitousek, 1997; Tilman et al., 1997; Symstad et al., 1998; Mulder et al., 2002; Spehn et al., 2005). However, a positive diversity-productivity correlation is not limited to the legume effect but has also been reported for systems without legumes (van Ruijven and Berendse, 2003; Marquard et al., 2009), thus indicating that plants can apprehend additional possibilities for nitrogen partitioning. For instance, it has been suggested that plant species differences in vertical root distribution (Parrish and Bazzaz, 1976; Yeaton et al., 1977) and activity might facilitate spatial N niching and indeed this process could be shown in the field (Veresoglou and Fitter, 1984; Mamolos et al. 1995). Though it is known that plants have the ability to take up amino acids as alternative N source in addition to mineral N enabling chemical N partitioning, investigations of this concept are scarce in the field of plant diversity research.

1.4 Methodological considerations

The two crucial points in measuring uptake of intact amino acids are (i) to proof uptake of intact amino acid molecules and (ii) to find a method which enables accurate quantification of amino acid uptake. While the uptake of nutrients from sterile hydroponic solutions can be quantified by recording concentration changes in the solution (Virtanen and Linkola, 1946; El-Naggar et al., 2009), this is not possible in natural ecosystems, were nutrients are released into and taken up from the soil solution at the same time. At this, the use of stable isotope tracers has been shown to be a powerful tool to investigate nitrogen uptake of plants (Owen and Jones, 2001; Bardgett et al., 2002; Bardgett et al., 2003; Cole et al., 2004; Dunn et al., 2006; Harrison et al., 2007). However, as for calculation of direct amino acid uptake, the exclusive use of ^{15}N labelled amino acids is not sufficient to proof direct uptake, as amino acids are known to be

mineralized within hours in soil (Jones, 1999; Jones and Hodge, 1999) and released mineral ^{15}N would be taken up by plants. The resulting plant ^{15}N enrichment would suggest direct uptake where indeed only mineral N has been taken up. Thus, Näsholm et al. (1998) applied dual labelled amino acids (^{15}N and ^{13}C) to soil of a boreal forest and measured the plants bulk enrichment for ^{13}C and ^{15}N. The authors found a high correlation between ^{13}C and ^{15}N enrichment in plant tissues and suggested that this is due to the uptake of both isotopes in form of the intact tracer molecule (Fig. S1A). As additional ^{15}N has been taken up in mineral form, the slopes of ^{13}C to ^{15}N enrichment found in plants were frequently lower than that of the original tracer molecule. By comparing the slope of plant enrichment to the tracer slope, the authors could calculate the relative amount of ^{15}N taken up in an intact form. Despite some flaws, this method is the most frequently used in investigations of direct amino acid uptake in natural systems. The most critical point of this method is the assumption that ^{13}C can only be taken up in the form of intact amino acids, i.e. changes in the ^{13}C:^{15}N enrichment in plants can only be caused by changes in ^{15}N uptake (Fig. S1A). However, some results in literature question this assumption, e.g. calculations of direct amino acid N uptake resulted in values of more than 100% intact uptake. A number of potential mechanisms were proposed causing this false estimation. First, plants have been shown to be capably of taking up a number of low molecular weight substances from soil, including organic acids (Kuzyakov and Jones, 2006; Biernath et al., 2008). One of the first steps in the degradation of amino acids in soil is decarboxylation (Kuzyakov, 1997), forming organic acids which thereafter can be taken up by plants. Any uptake of these ^{13}C labelled tracer fragments would lead to an overestimation of direct amino acid uptake (Fig. S1B). Second, if intact tracer amino acids are taken up, these can be subject to root internal deamination. If the released ammonium is transferred to other amino acids and transported to the shoot, this would result in a relative enrichment of ^{13}C compared to ^{15}N in roots, again resulting in a false estimation of intact tracer N uptake for the roots (Fig. S1C). In contrast, an underestimation of direct amino acid uptake would be the result of plant internal decarboxylation of amino acids (Fig. S1D). When glycine is used as tracer, a fourth process influencing calculation has to be considered: Two glycine molecules can be transferred to one molecule of serine, ammonium and CO_2 in soil via the microbial glycine-decarboxylase pathway (Oliver, 1994). This changes the original ^{13}C:^{15}N enrichment from 2:1 (glycine) to 3:1 (serine) and thus plant uptake of these serine molecules would result in an enrichment ratio of 3:1 instead of 2:1 in the case of

glycine. As a result, intact amino acid N uptake would be calculated to 150 %. However, bulk isotope measurements are not able to differentiate between the uptake of tracer fragments or tracer molecules transformed before uptake and the actual uptake of intact amino acids.

Beside this considerable error rate of bulk measurements in combination with the Näsholm calculation method (Näsholm et al., 1998) in soils with significant microbial activity, a more general methodological constraint is the significant detection of ^{13}C enrichment in plant material: This can be especially difficult when low levels of amino acid tracer are used in order to avoid alteration of the size of the natural amino acid pool in soil. Several studies found considerable amounts of ^{15}N in plant shoots while significant ^{13}C enrichment was not detected (Lipson and Monson, 1998; Hodge et al., 1999; Hodge et al., 2000b). Apart from decarboxylation processes of amino acids in the plant root, this mismatch is mainly related to a stronger dilution of the ^{13}C label compared to ^{15}N. On the one hand, plant C content is much higher than plant N content (45 – 50 % C compared to 3 – 5 % N in the dry weight) and on the other hand the natural ^{13}C content of plants is higher (ca. 1.08 % for C3 plants) compared to ^{15}N, leading to a dilution of ^{13}C that is 60 – 150 times higher than that of ^{15}N (Näsholm and Persson, 2001b).

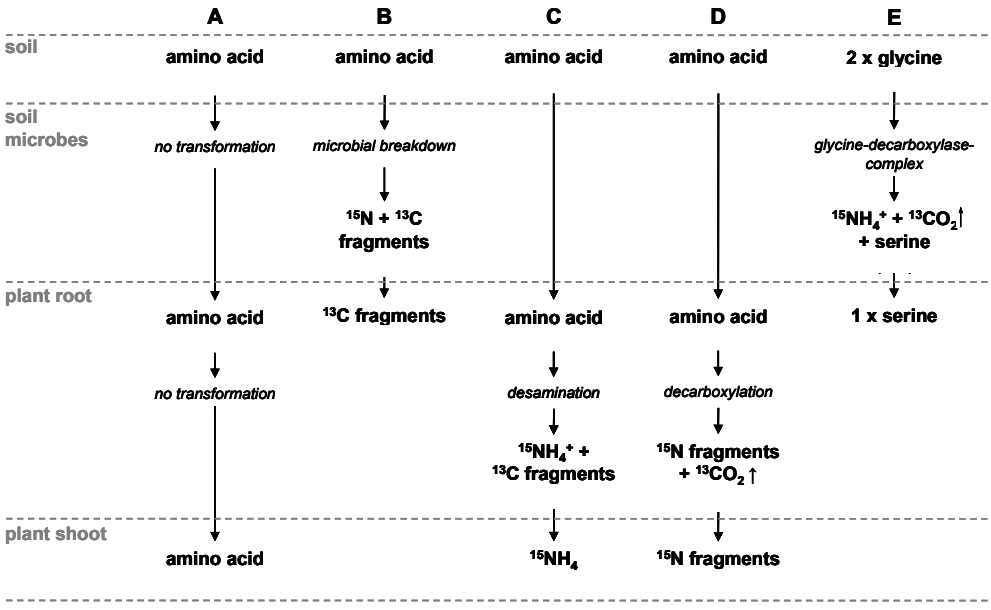

Figure S1: Possible mechanisms influencing the ^{13}C:^{15}N enrichment in plant tissue after application of dual labelled amino acids.

The use of compound specific isotope (CSI) measurements is assumed to overcome all these methodological constraints as this method enables ^{13}C enrichment measurement in the specific amino acids used as tracer (Persson and Näsholm, 2001; Jones et al., 2005a). In addition, CSI measurements would enable the use of smaller tracer amounts,

as tracer C is only diluted by the original amino acid pool of plants but not by the whole plant C pool and due to the higher sensitivity of CSI compared to bulk measurements (Glaser, 2005). Despite these advantages, CSI measurements so far have only been used for proofing direct amino acid uptake (Persson and Näsholm, 2001) but not for quantification.

1.5 Objectives

Previous investigations have shown the possible importance of amino acid uptake for the plant N nutrition in N-limited systems. In addition, biodiversity research over the last two decades proved that complementarity and niching in resource acquisition can explain the positive plant diversity-productivity correlation. In this context the use of amino acid uptake as alternative N source might be a possible mechanism to reduce plant interspecific competition for N, increase the community's nitrogen use efficiency and stimulate the productivity of plant communities with increasing plant diversity. However, the importance of amino acid uptake in this context can only be quantified correctly if the reliability of the used labelling and measuring methods are proven. The methodological objectives of my dissertation therefore were

(1) to investigate the influence of amino acid concentrations in soil and used tracer amounts on the direct amino acid uptake by plants,
(2) to find the optimal sampling time after labelling and optimize the sampling procedure for root samples,
(3) to develop a method for compound specific measurements of ^{13}C and ^{15}N values of individual amino acids,
(4) to clarify whether the quantitative differences in the calculation of amino acid uptake between conventional bulk and compound specific isotope (CSI) measurements justify the use of costly CSI measurements.

Thereafter, the optimized labelling and measuring techniques should be used to

(5) investigate how increasing plant diversity alters the use and importance of amino acids for the plant N nutrition,
(6) elucidate the influence of plant diversity on the competition of plants and microbes for amino acids,
(7) show whether a change in the plant to microbial competition is accompanied by a change in the activity of single microbial groups with respect to amino acid metabolisation or is due to a change of the whole microbial community structure.

2 Greenhouse experiments

To address the effect of amino acid concentrations and tracer amounts on plant amino acid uptake, a greenhouse experiment was conducted using six week old potted *Anthoxanthum odoratum* plants as model plant. Second, the experiment should help to find the optimal tracer amount with respect to minimum tracer use and maximum accuracy of the detected ^{13}C enrichment. And third I wanted to define the optimal sampling time after tracer application to measure amino acid uptake. We used mixtures of four dual labelled (^{13}C and ^{15}N) amino acids (glycine, valine, lysine and tyrosine) that were directly injected into the soil. These amino acids were chosen to cover a wide range of chemical characteristics with respect to C:N ratio, sorption reaction in soil, microbial degradability and molecule size. In the first treatment, four different tracer amounts were applied (0.002, 0.025, 0.13, 2.5 µg N*g soil^{-1}) and samples (shoot and root) were taken 24 h after application to investigate the effect of tracer amounts on amino acid uptake. In a second treatment, a constant tracer amount was applied, but samples were taken after different time intervals after labelling (2, 4, 8, 16, 24, 48 and 96 h) to find the optimal sampling time with the highest amino acid uptake rates.

A second greenhouse experiment aimed at investigating if plant uptake of inorganic carbon derived from amino acid tracer mineralization in soil is sufficient to explain differences between bulk and compound specific measurements, as investigated in the first field experiment (see 4.1). Maize plants were grown in rhizosphere tubes as described in Biernath et al. (2008) for two weeks. Labelled glycine (^{13}C and ^{15}N, or ^{14}C) or labelled bicarbonate (^{13}C or ^{14}C) was added to the soil in the tubes and plant tissues were sampled after 24 h.

All other aforesaid objectives of the Phd-thesis were investigated on the field site of the Jena Experiment which will be described in the following.

3 Field experiments

3.1 Experimental site

All field experiments were conducted at the field site of the *Jena Experiment* which is located on a flood plain of the the Saale river near Jena, Germany (11°34'60" East; 50°55'60" North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was intensively fertilized during the last 40 years of agricultural utilisation until 2002 when the *Jena Experiment* started.

The experimental setup consists of 92 plots (20 x 20 m in size) differing in number of plant species and functional plant groups. As these plots are only used for non-destructive measurements, all plots have small replicates (3.5 x 3.5 m in size) which were used for the investigations of the present PhD-thesis. Plots were established from seeds using a pool of 60 common European grassland species. These were comprised of four functional plant groups, namely grasses, small herbs, tall herbs and legumes. Plant diversity was altered from 1 to 60 plant species and from 1 to 4 functional plant groups. The plots were randomly distributed across four blocks that were arranged parallel to the Saale river (Fig. S2). This was done to compensate gradients of soil parameters like pH, soil density and soil texture, which were oriented perpendicular to the Saale river.

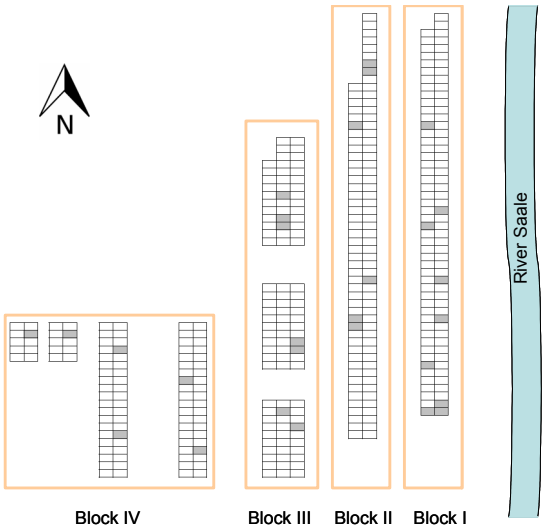

Figure S2: Design of the Jena Experiment field site; rectangles represent small replicate plots (5 x 5 m) and grey rectangles give the position of plots used for investigations of amino acid uptake

3.2 Experiments on methodological considerations

The main objective of the first field experiment was the investigation of methodological differences in amino acid uptake calculations based on bulk and CSI measurements. Beside this, the applicability of the optimized labelling and sampling techniques as derived from the greenhouse experiment should be tested in the field. For this purpose a monoculture of *Plantago lanceolata* was established on the field site of the *Jena Experiment* by seeding and planting of 8 weeks old seedlings in spring 2006. Special care had to be taken to avoid tracer contamination of the surrounding field, were background ^{15}N and ^{13}C values were measured by other members of the *Jena Experiment*. Therefore, I took 5 undisturbed soil monoliths from the monoculture, in August 2006,

one week before sampling started, by pressing a poly ethylene tube (30 cm diameter) into the soil. Soil cores were then brought to a 35 cm deep water proof trench on the field site were they were installed in a way that free drainage was ensured. The free space between the tubes containing the soil cores and the surrounding soil was filled with soil to allow for natural soil temperature dynamics. We applied four dual labelled amino acids (glycine, valine, tyrosine, lysine) by injecting amino acid solutions into the soil to a depth of 5 cm. Four replicate soil cores received labelled amino acids, while the fifth served for evaluation of ^{13}C and ^{15}N background values, thus only receiving unlabelled amino acids. 24 h after label application shoot and root biomass was sampled, washed in the case of roots, frozen in liquid N_2 and stored frozen until further analysis.

3.3 Experiment on amino acid uptake in a plant diversity gradient

The second field experiment had three objectives:

(1) to investigate changes in the amount of plant amino acid uptake and its importance for the plant N nutrition with respect to changing plant diversity,

(2) to depict the importance of microbial and plant competition for amino acids and soil N and investigate changes of this competition status with changing plant diversity,

(3) to determine the effect of plant diversity on structure and activity of the soil microbial community with respect to amino acid metabolisation.

The experiment was conducted in August 2007 on small replicate plots of the Jena Experiment. Due to the ability of legumes to fix N_2 from the air, I assumed that amino acid uptake is of minor importance for this functional plant group and therefore only plant mixtures without legumes were investigated. Overall a number of 28 plots, representing plant diversity levels of 1 (SR1), 2 (SR2), 4 (SR4), 8 (SR8) and 16 (SR16) plant species were used. Each diversity level was present in 6 fold replication with the exception of SR16 with only four replicates. The mixtures were established from a pool of 48 common European grassland species that had been assigned to three different functional groups: 16 grasses, 12 small herbs and 20 tall herbs. One year before the experiment started, one individual of each of three phytometer species (*Plantago lanceolata, Geranium pratense* and *Festuca pratense)* were planted in the centre of three rings located in one quarter of each plot. This was done as it had been shown in literature that the uptake potential for amino acids of different plant species widely varies (Kielland, 1994; Weigelt et al., 2003; Weigelt et al., 2005). Changes in the amino acid uptake between plots of different biodiversity might therefore be rather due to changes in sampled spe-

cies than due to the effect of altered biodiversity. The sampling of plant species that are present in all plots (phytometers) removes this effect, leaving plant diversity as the sole manipulated variable. Four weeks before labelling started, all plots were equipped with three polyethylene tubes (30 cm diameter), which enclosed the three phytometer rings. The tubes were installed to a depth of 30 cm, leaving a ring of 10 cm aboveground. They impeded lateral dispersal of applied tracer and enabled the measurement of gas efflux by closing the upper part of the tube for short time intervals. Two of the three rings were used for application of labelled amino acids, while the third ring served as background. Intact amino acid uptake was only measured in one labelling ring while the other ring was used for investigation of the long term turnover of soil amino acids, including gas efflux measurements. However, the latter one is not in the scope of this PhD-thesis and therefore will not be presented here. In contrast to the greenhouse experiments and the first field experiment, we replaced tyrosine and lysine by phenylalanine due to incomplete peak separation of these two amino acids in CSI measurements. Sampling and sample storage was done as outlined in section 3.2 with the exception that soil samples for estimation of microbial biomass were stored at 5 °C until further analysis.

4 Analytical methods

All plant samples were freeze dried and ground to fine powder before analysis, while soil samples were only sieved to < 2 mm followed by freeze drying. Unless otherwise noted, amount and isotopic signature of all following chemical compounds were analysed on a gas-chromatograph (Trace GC 2000, Thermo Finnigan) coupled to an isotope ratio mass spectrometer (IRMS; Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany) via a combustion interface (GC-C-IRMS).

4.1 Amino acids

Free and protein-bound amino acids were extracted from plant tissues by means of hot acidic hydrolysis. Extraction, purification and derivatization of amino acids followed the procedure described by Amelung & Zhang (2001). Free soil amino acids were extracted with hot $CaCl_2$ solution as outlined in Houba et al. (1986) and Mengel et al. (1999). As high cation concentrations in soil disturbed the derivatization procedure of Amelung & Zhang., this was replaced by the use of the amino acid derivatization kit EZ:faast$^{®}$ (Phenomenex, Torrance, CA, USA) in the case of free soil amino acid analy-

sis. Recovery of both methods was calculated using Norvaline and trans-4-(aminomethyl) cyclohexane carboxylic acid as first and second internal standard, respectively. Along with the samples, standard mixtures of 12 amino acids were derivatized and used for amount and isotope ratio calibration (see below).

4.2 Phospholipid fatty acids (PLFA)

Extraction and purification of phospholipids followed the procedure as outlined in Frostegard et al. (1991). After purification, phospholipids were saponified to free fatty acids and transformed to fatty acid methyl esters following the derivatization procedure of Knapp (1979). Standard mixtures of 21 fatty acids were derivatized parallel to soil samples.

4.3 Soil microbial biomass

Microbial biomass was extracted from soil within one week after sampling by means of the chloroform fumigation (CFE) method (Brookes et al., 1985). Microbial C and N amounts were quantified in the resulting liquid extracts using a liquid total carbon / nitrogen analyser (DIMA TOC-100, Dimatec, Essen, Germany) while the isotope signature was measured in the dried extracts using an elemental analyser (EA) coupled to an IRMS (Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany).

4.4 Mineral soil N

Mineral N (NH_4^+ and NO_3^-) was extracted from soil sieved to < 2 mm using 0.5 M KCl. Nitrate and ammonium contents thereafter were measured using a continuous flow analyser (Bran&Luebbe, Norderstedt, Germany).

4.5 Bulk isotope measurements

Bulk isotope composition (^{13}C and ^{15}N) was measured in plant tissues, microbial biomass extracts and in underivatized amino acid and PLFA standards using an EA-IRMS (Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany). Measured δ-values were calibrated against international standards using Sucrose (CH_6, IAEA, Vienna, Austria, $\delta^{13}C = -10.47$ ‰), $CaCO_3$ (NBS 19, Gaithersburg, USA, $\delta^{13}C = +1.95$ ‰) and Acetanilid (Carlo Erba Instruments, Milan, Italy, $\delta^{13}C = -34.09$ ‰) in the case of $\delta^{13}C$ and using two types of $(NH_4)_2SO_4$ (IAEA-N1, $\delta^{15}N = 0.36$ ‰ and IAEA-N2, $\delta^{15}N = 20.3$ ‰), KNO_3 (IAEA-N3, $\delta^{15}N = 4.7$ ‰) and two ^{15}N-enriched forms of $(NH_4)_2SO_4$ (USG-26, IAEA, $\delta^{15}N = 53.7$ ‰ and USG-32, IAEA, $\delta^{15}N = 180$ ‰) for $\delta^{15}N$ measurements. The ^{14}C activity of plant tissues derived from the second greenhouse experiment were meas-

ured using a Packard model 307 sample oxidizer (Packard Instrument Company, Meriden, CT, USA)

4.6 Compound specific measurements

The isotope signature and amount of amino acids and PLFA were measured using a GC-C-IRMS as described in the beginning of this section. Detailed information on the instrument setup including the optimized GC settings can be found in Study 2. As derivates of amino acids and PLFA were measured, the observed δ-values included the signature of derivatization C. To correct for this effect and reference the δ-values to international standards at the same time, I used the correction procedure of Glaser & Amelung (2002).

4.7 Calculation of tracer uptake

The calculation of tracer N and C uptake into a compartment (plants, microbial biomass, PLFA, or amino acids) was based on the ^{13}C and ^{15}N enrichment of the specific compartment, calculated as the difference between labelled and unlabelled treatment. In this, the calculation followed a two pool dilution model as suggested by Gearing et al. (1991). For experiments were natural soil amino acids and soil N concentration differed between treatments (Study 2) I also corrected for the effect of different tracer dilution by these soil pools according to Buchmann et al. (1995).

5 Results and Discussion

5.1 Effect of soil amino acid concentration on direct amino acid uptake (Study 1)

The use of different tracer amounts to simulate different soil amino acid concentrations resulted in three central findings: The lowest application amount of 0.002 µg N*g soil^{-1} did not result in significant ^{13}C enrichment of plant tissues. Therefore, uptake of tracer N as intact tracer molecule (direct uptake) could not be calculated und was not included in further calculations. For all other treatments ^{13}C enrichments were significant and the amount of tracer significantly influenced the total uptake of tracer ^{15}N (direct uptake + uptake of tracer derived mineral ^{15}N) as well as direct tracer N uptake, both relative to the applied tracer amount. Relative total ^{15}N uptake increased in average from 13 to 28 % with increasing tracer amount. This trend was true for all used amino acids though it has to be noted that glycine frequently showed highest uptake values. In contrast to this, the relative direct ^{15}N uptake decreased in average from 5.4 to 1.5 %

with increasing tracer amount. This trend was significant for all amino acids, with glycine having the lowest uptake values of all used amino acids.

From these findings I concluded that the plant uptake of intact amino acids is negatively affected by high N-availability in soil due to plant internal down regulation of amino acid transporters under conditions of high N availability in soil as already suggested by Persson & Näsholm (2002; 2003). This finding implicates:

(1) High tracer amounts increase the N availability in soil due to mineralization of tracer N. As a result, this lowers amino acid uptake by plants and thus reduces the relative importance of direct amino acid uptake for the plant N nutrition. Therefore, tracer amounts need to be minimized as far as possible to ensure minimal impact on the N status of the plant-soil system.

(2) Due to plant internal regulation of amino acid uptake, an increasing importance of this N source for the plant N nutrition has to be expected for systems with low N mineralization rates or high N competition between plants or between plants and microbes.

Along with these published results, the experiment enabled me to define the optimal sampling time for the investigation of amino acid uptake. This was set to 24 h after application, as ^{13}C enrichments in shoot and root tissues were highest after this time period. This enabled highest accuracy for the detection of ^{13}C enrichment while using minimum tracer amounts.

5.2 Suitability of bulk vs. CSI measurements for accurate quantification of amino acid uptake (Studies 2, 3)

The comparison between both methods was based on differences between measured ^{13}C enrichments, as calculation of direct amino acid uptake using the common bulk method (Näsholm et al., 1998) is based on ^{13}C enrichment.

^{13}C enrichment of individual amino acids in shoot and root samples as derived from CSI measurements was limited to the amino acids used as tracer. This indicates that no additional ^{13}C has been incorporated in the plant amino acid pool via the uptake of tracer ^{13}C fragments derived from microbial tracer mineralization in soil. Thus, CSI measurements are unlikely to overestimate amino acid uptake. While results from bulk measurements also suggested uptake of intact amino acids due to a high correlation between ^{13}C and ^{15}N enrichment in plant tissues (Näsholm et al., 1998), ^{13}C enrichment differed significantly from CSI measurements. In detail, bulk ^{13}C enrichments were 8, 5,

1.6 and 6 times higher for fine roots, storage roots, shoot and the whole plant, respectively (Fig. S3). This suggests that plants are capable to take up tracer fragments or inorganic C derived from tracer mineralization as already suggested by Jones et al. (2005a) and Rasmussen & Kuzyakov (2009). However, bulk isotope measurements are not able to separate this from the uptake of intact amino acids thus leading to an overestimation of amino acid uptake.

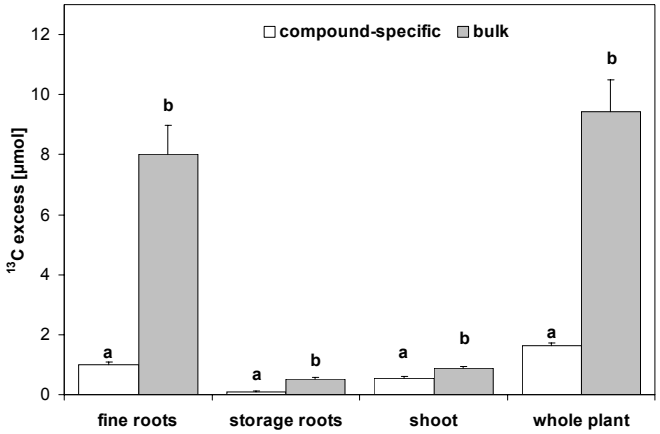

Figure S3: ^{13}C enrichment measured via bulk or CSI measurement of the target amino acids. Bars show mean values of four field replicates ± standard error; different lower case letters show significant differences between both measurements for one plant tissue ($p<0.05$).

In addition to this, I used CSI measurements to investigate the ^{15}N enrichment of individual amino acids. I found that all 12 evaluated amino acids were significantly enriched in ^{15}N and that the overall ^{15}N enrichment in the amino acid pool was higher than the corresponding ^{13}C enrichment. As ^{15}N enrichment in the original tracer molecules was lower, this indicated uptake of tracer derived mineral ^{15}N. The mineral ^{15}N was rapidly transferred to newly synthesised amino acids and thus ^{15}N enrichment of amino acids does not indicate direct amino acid uptake, which proofs the need of ^{13}C CSI measurements of amino acids.

The application of equal amounts of amino acids or bicarbonate to potted maize plants in Study 3 resulted in equal bulk ^{13}C enrichments for both treatments. This indicates that the plants potential to take up and incorporate bicarbonate via dark fixation (Vuorinen et al., 1989; Vuorinen et al., 1992) is equal to their amino acid uptake potential. As $^{13}CO_2$ originating from microbial tracer mineralization can be dissolved in soil solution being transformed to bicarbonate (Stumm and Morgan, 1996), bicarbonate uptake could significantly contribute to the plants bulk ^{13}C enrichment. This would constrain correct quantification of amino acid uptake when using bulk measurements, espe-

cially in soils were high pH values boost the formation of bicarbonate as in the case of the *Jena Experiment*.

I therefore conclude that

(1) Bulk isotope measurements are not suitable for the correct quantification of amino acid uptake due to the unconsidered uptake of tracer C fragments and should thus be replaced by CSI measurements.

(2) CSI measurements of ^{13}C enrichment in amino acids are an accurate tool for the correct quantification of direct amino acid uptake.

(3) CSI measurements offer the possibility to evaluate the effect of incorporation of tracer C fragments on the accuracy of calculated direct amino acid uptake amounts by measuring the ^{13}C enrichment of non tracer amino acids in plant tissues.

5.3 Soil amino acid fingerprint (Study 4)

As demonstrated in study 1, the amino acid concentrations in soil are likely to influence the uptake of amino acids by plants. Therefore, changes of amino acid concentrations between plots used for quantification of amino acid uptake in studies 5 and 6 were investigated. The size of the free soil amino acid pool showed a rising trend from 538 to 696 nmol * g^{-1} soil with increasing plant diversity, though this trend was not significant. It has been assumed that plants produce more root exudates under conditions of high N competition to stimulate microbial N mineralization (Lemaire and Millard, 1999; Raynaud et al., 2008). Roscher et al. (2008) showed that there is increased competition for nitrogen with increasing diversity due to higher plant productivity in the *Jena Experiment* and thus exudation of amino acids as one of the main constituents of root exudates (Kraffczyk et al., 1984; Farrar et al., 2003) should increase. However, it is also known that the bulk of the microbial soil community is feeding on root exudates (Haller and Stolp, 1985) and as we found an increasing microbial abundance with increasing plant diversity, the higher microbial turnover of exudates and thus amino acids might have led to an insignificant increase free soil amino acids.

The free soil amino acid pool was dominated by phenylalanine and histidine, irrespective of plant diversity. However, the contribution of all other amino acids changed between diversity levels, enabling us to separate the single diversity levels by means of a discriminant analysis. The cause for this changing amino acid fingerprint can be due to two factors: Plants are known to widely differ in the composition of their root exudates (Marschner et al., 2004) and thus the changing amino acid fingerprint in soil might reflect changes in the plant species composition. In addition, Hertenberger et

al. (2002) demonstrated that microbial communities differing in metabolisation characteristics are able to change the composition of amino acids in soil. As I found strong differences in the composition of the microbial community (see 5.5) between diversity levels, this indeed might be the dominant process forming the soil amino acid fingerprint. Thus, it has to be expected that the plant to microbial competition for amino acids changes with changing plant diversity, influencing the plant amino acid uptake.

5.4 Influence of plant diversity on direct amino acid uptake by plants and the importance of microbial competition (Study 5)

We found a positive plant diversity effect on plant productivity, increasing plant aboveground biomass production from 80.9 g m^{-2} to 200 g m^{-2}. This was accompanied by an increase of N stored in the whole plant biomass from 2.52 g m^{-2} to 3.34 g m^{-2} for monocultures (SR1) and 16 species mixtures (SR16), respectively. While the positive effect of plant diversity on plant productivity is a well described phenomenon (Tilman et al., 1996; Hector et al., 1999; Loreau et al., 2001; Hooper et al., 2005), the mechanisms how plants manage to be more productive are still under debate. With respect to the increased N storage in plant biomass as observed in this study it is especially of relevance, how plants manage to acquire more N as this is one of the main growth limiting nutrients (Vitousek and Howarth, 1991). A higher N supply of plants could either be due to increased N uptake efficiency of plants or due to higher N availability in soil. Microbes are known to be important competitors for N in soil (Hodge et al., 2000a) and thus, any change in this competition would alter the N availability in soil.

The competition for N was investigated using amino acid tracer as amino acids are one of the main precursor substances for mineral N and are available for plants and microbes. Tracer uptake data showed that the microbial community, which grew in size with increasing diversity, took up 54 % of the applied tracer N in average und thus microbes were superior competitors for amino acid derived N. However the amount of microbial total tracer N uptake (intact + mineral N) was not influenced by plant diversity. In contrast, plants were only able to take up 2.68 % of applied tracer N in average and this relative amount decreased from 3.75 % to 1.53 % with increasing diversity. Besides plant diversity, most of these variations (20 %) were explained by the negative effects of a growing microbial community and microbial total N uptake, while much less (9 %) were explained by plant parameters such as root density, aboveground biomass or plant N concentration. Thus, total plant N uptake (intact + mineral N) was rather controlled by microbial competition than by the plant-regulated uptake potential.

In contrast to this, plants increased the uptake of intact amino acids with increasing diversity (Figure S4). This is surprising as 48 % of the tracer C was taken up by microbes and additional 50 % were respired in average, indicating a superior competitive power of microbes for tracer uptake. However, within these boundaries that are defined by microbial competition, plants were able to successfully adjust amino acid uptake to their N demands. This is indicated by the fact that most of the variations (21 %) of direct amino acid uptake were explained by plant parameters. Among those, shoot biomass and root density had a positive effect, i.e. amino acid uptake depended on the size of sink organs for N and the potential number of amino acid transporters per gram soil.

Second, the plant N concentration had a negative effect on direct amino acid uptake, i.e. plants with a high N status took up less intact amino acids than did plants with a low N nutrition status. This control mechanism corroborates results found in study 1. Thus, the increased amino acid uptake has to be seen as an N partitioning strategy to compensate decreasing mineral N uptake due to increased microbial competition for N. In doing so, plants bypass the microbial mineralization of amino acid N and lower the retention potential of the microbial biomass for amino acid N. This N partitioning has already been assumed to be of importance (Hooper et al., 2005; and citations therein) and has been described recently by von Felten et al. (2009), though these authors were not able to proof uptake of intact amino acid by means of CSI measurements.

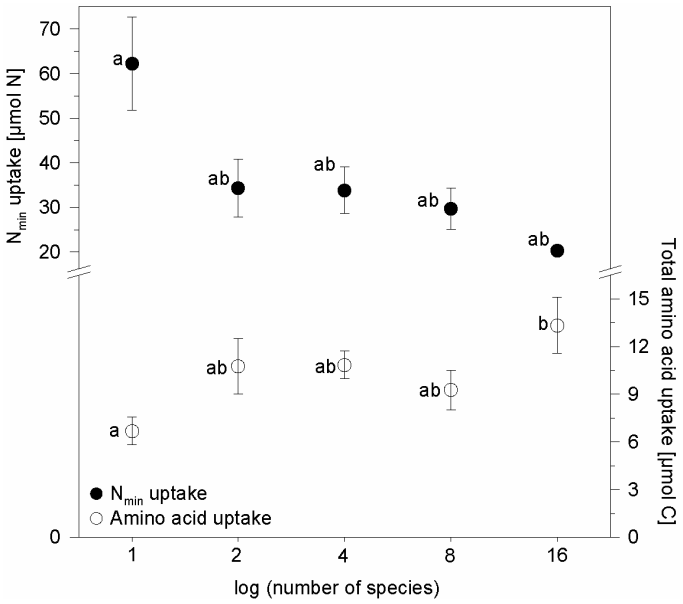

Figure S4: Mineral N uptake and amino acid uptake of plants depending on plant species number.

To conclude these results:

(1) Microbes were shown to be superior competitors for N, irrespective of plant diversity and highly affect plant N uptake.

(2) Plants took up less N with increasing plant diversity which was mainly due to an unspecific diversity effect but also due to microbial competition.

(3) As reaction to lower total plant N uptake caused by lower N_{min} uptake, plants increased their amino acid uptake which was shown to be controlled by plant internal regulation mechanisms based on the N nutrition status of the plants.

(4) As a result, the contribution of amino acid N uptake to the total N uptake increased from 1.47 to 7.04 % with increasing plant diversity, indicating intensified N partitioning of plants.

It has to be noted that these results reflect the competition status for N between microbes and plants in the short term (24 h). As suggested by Hodge et al. (2000a) this might change dramatically in favour of the plants in the long term due to the high turnover rates of microbes. As this turnover rate might change with plant diversity, I investigated changes of the microbial community structure and its metabolic activity in the plots of the present study as described in the following section.

5.5 Changes in microbial community structure and metabolic activity (Study 6)

Plant diversity had a significant effect on the structure of the microbial community, increasing the microbial diversity in soil as indicated by the Simpson index. With increasing diversity, the relative abundance of fungi, one group of gram positive bacteria (G^+1) and protozoa increased. Fungi and G^+1 are known to be involved in decomposition of complex organic materials (Poll et al., 2008) and SOM (Kramer and Gleixner, 2006), respectively. As increasing plant diversity is accompanied by increasing chemical diversity of litter input due to species specific differences in litter composition (Gransee and Wittenmayer, 2000; Eskelinen et al., 2009), this ought to stimulate fungal growth by additional possibilities for niching (Hooper et al., 2000). At the same time, higher plant productivity with increasing diversity as found in this investigation was shown to result in higher SOM contents (Steinbeiss et al., 2008), thus boosting growth of G^+1. Protozoa which nearly doubled their abundance with increasing plant diversity mainly feed on prokaryotes (Kuikman et al., 1990) and the growth of these in return is known to be stimulated by increasing root exudation. Though we have not measured root exudation, there is strong evidence that plants increase their root exudation under conditions of higher N competition (Lemaire and Millard, 1999; Raynaud et al., 2008) which has been shown to occur in our experiment (Roscher et al., 2008 and see also 6.4). As protozoa boost the turnover of the prokaryotic community (Kuikman et al., 1990), we have to expect increased microbial turnover with increased plant diversity.

Amino acid uptake was dominated by prokaryotes which are known to mainly feed on low molecular weight substances (Haller and Stolp, 1985) and this uptake in-

creased with increasing plant diversity. At the same time we found higher tracer uptake of protozoa feeding on these prokaryotes, which indicates a higher flow of tracer through the microbial food web suggesting a higher turnover of the microbial community for higher plant diversity levels. This was corroborated by higher deaminase activity in soil as also found by Badalucco et al. (1996) for soils with high protozoan activity.

The positive effect of plant diversity on microbial turnover and deaminase activity via reorganisation of the microbial community as found in our investigation is thus likely to increase N availability for plants (Zak et al., 2003), resulting in a positive feedback of plant diversity on plant productivity.

6 Conclusions

Preliminary investigations in the present work showed that uptake of intact amino acids is reduced under high soil amino acid concentrations. This was mainly due to the down regulation of amino acid transporters under conditions of higher soil N availability as induced by higher absolute mineralization of soil amino acids. The detected changes of the soil amino acid fingerprint in grassland communities differing in plant diversity is thus likely to cause differences in the direct uptake of amino acids.

However, the present investigations proofed that the actual importance of amino acid uptake can only be estimated using compound specific isotope measurements as commonly used bulk measurements are not able to separate direct uptake of tracer molecules from uptake of tracer fragments and thus, are likely to overestimate direct amino acid uptake. In this context, results of amino acid uptake studies using bulk isotope measurements in soils with significant microbial activity need to be questioned. In addition, the dilution of tracer amino acids by natural soil amino acids needs to be considered to depict the plants real actual amino acid uptake and estimated its ecological impact in soils differing in natural amino acid concentrations.

In doing so, N-nutrition patterns of plants growing in plots of different plant diversity revealed an increased uptake of intact amino acids with increasing diversity. At the same time, the plants mineral and overall N uptake decreased, which apart from a pure, unspecific diversity effect was mainly due to the superior competitive power of microbes for N acquisition in the short term. In this, the contribution of amino acids to the plants overall N supply increased from 1.47 to 7.04 %, thus indicating increased niching in N acquisition as a reaction on increasing N competition with increasing plant

diversity. These findings are strong evidence for a stimulation of plant productivity due to increased N source partitioning. However, the results on N competition between microbes and plants only reflected a short time window (24 h) and are known to change in favour of the plants in the long term due to the high turnover of microbes.

In this context, results from PLFA analysis suggest an increasing turnover of the prokaryotic community due to stimulation by increased root exudation and increased grazing by protozoa. This was corroborated by an increased microbial turnover of amino acids and increased deaminase activity and thus ought to result in a higher loss of microbial N to plants. Therefore the bottom up control of the microbial community structure by plant diversity ought to stimulate N availability for plants in soil. This positive feedback mechanism, in combination with increased N-source partitioning of plants might be a possible model to explain the positive effect of plant diversity on plant productivity.

Though the overall contribution of amino acid uptake to the plant N nutrition was low, this does not necessarily imply that amino acid uptake generally is of inferior importance for plants. The present investigations rather indicate that plants improve their N supply in the short term by an increased amino acid uptake with increasing plant diversity and in the long term by stimulating microbial turnover. As the latter one comes at the price of increased microbial competition for N in the short term, amino acid uptake has to be seen as a strategy to compensate this negative side effect, thus leading to an overall higher N supply of plants with increasing plant diversity. Ongoing investigation of the fate of the applied amino acid tracer in the long term are intended to proof this mechanism. Thus, amino acid uptake can be an important part of the plants' corporate strategy to improve N supply in situations of increased N competition, though its contribution to the overall N uptake might be small at first glance. In this context it should be of specific interest of further investigations to elucidate the role of direct amino acid uptake for the persistence of subordinate plant species in highly productive plant communities.

7 Contribution to the included manuscripts and publications

The two publications and four manuscripts included in the PhD thesis were prepared in cooperation with various coauthors. The coauthors listed in these manuscripts contributed as follows:

Study 1

Status: Published in Environmental and Experimental Botany, 2009, Vol. 66, Issue 2, page 145 – 152

Contributors:

Leopold Sauheitl: 86 % (Experimental design, accomplishment of experiments, chemical analysis, data preparation, preparation of manuscript)

Bruno Glaser: 7 % (Discussion on experimental design and results, comments to improve the manuscript)

Alexandra Weigelt: 7 % (Discussion on experimental design and results, comments to improve the manuscript)

Study 2

Status: Published in Rapid Communications in Mass Spectrometry, 2009, Vol. 23, page 3333 – 3342

Contributors:

Leopold Sauheitl: 77 % (Experimental design, accomplishment of experiments, chemical analysis, data preparation, preparation of manuscript)

Bruno Glaser: 10 % (Discussion on experimental design and results, laboratory support during CSI measurements, comments to improve the manuscript)

Alexandra Weigelt: 13 % (Discussion on experimental design and results, support during field work, comments to improve the manuscript)

Study 3

Status: Submitted to Soil Biology and Biogeochemistry; date: 12.08.2009

Resubmitted on 13.10.2009

Contributors:

Jim Rasmussen: 50 % (Experimental design, accomplishment of experiments, chemical analysis, data preparation, preparation of manuscript)

Leopold Sauheitl: 25 % (Experimental design, support during experiment, support during data preparation, comments to improve the manuscript)

Jørgen Erikson: 10 % (Support during statistical analysis, comments to improve manuscript)

Yakov Kuzyakov: 15 % (Experimental design, support during experiment, comments to improve the manuscript)

Study 4

Status: Submitted to Plant and Soil; date: 20.10.2009

Contributors:

Leopold Sauheitl: 80 % (Experimental design, accomplishment of experiments, chemical analysis, data preparation, preparation of manuscript)

Bruno Glaser: 5 % (Discussion on experimental design and results, comments to improve the manuscript)

Michaela Dippold: 5 % (Support in the field and during laboratory work, comments to improve manuscript)

Katharina Leiber: 3 % (Support during laboratory work, comments to improve manuscript)

Alexandra Weigelt: 7 % (Discussion on experimental design and results, support during field work, comments to improve the manuscript)

Study 5

Status: To be submitted to Journal of Ecology

Contributors:

Leopold Sauheitl: 65 % (Experimental design, accomplishment of experiments, chemical analysis, data preparation, preparation of manuscript)

Michaela Dippold: 8 % (Support in the field, laboratory work on microbial biomass)

Bruno Glaser: 8 % (Discussion on experimental design and results, comments to improve the manuscript)

Romain Barnard: 3 % (Support during field and laboratory work for microbial biomass, comments to improve manuscript)

Nina Buchmann: 3 % (Discussion on experimental design, comments to improve manuscript)

Alexandra Weigelt: 13 % (Discussion on experimental design and results, support during field work, comments to improve the manuscript)

Study 6

Status: To be submitted to Journal of Ecolology

Contributors:

Leopold Sauheitl: 54 % (Experimental design, accomplishment of experiments, support during laboratory work, data preparation, preparation of manuscript)

Michaela Dippold: 30 % (Support in the field, laboratory work of PLFA analysis, data preparation, comments to improve manuscript)

Bruno Glaser: 8 % (Discussion on experimental design and results, support during laboratory work, comments to improve the manuscript)

Nina Buchmann: 2 % (Comments to improve manuscript)

Alexandra Weigelt: 6 % (Discussion on experimental design and results, support during field work, comments to improve the manuscript)

Literature

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Direct Amino Acid Uptake by Plants

related to Grassland Diversity

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methodological and ecological Investigations

II) Cumulative Publications and Manuscripts

Study 1: Uptake of intact amino acids by plants depends on soil amino acid concentrations

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Abstract

Studies in different ecosystems have shown that plants take up intact amino acids directly but little is known about the influence of free amino acid concentrations in the soil on this process. We investigated the effect of three different soil amino acid N concentrations (0.025, 0.13 and 2.5 µg N g^{-1} soil) on direct uptake of four dual labelled (^{15}N, ^{13}C) amino acids (glycine, tyrosine, lysine, valine) in a greenhouse experiment using *Anthoxantum odoratum* as a model plant.

Our results revealed that 8 – 45 % of applied ^{15}N were incorporated into plant root and shoot tissue 48 h after labelling. Additional ^{13}C enrichment showed that 2 – 70 % of this incorporated ^{15}N was taken up as intact amino acid. Total ^{15}N uptake and ^{15}N uptake as intact amino acids were significantly affected by soil amino acid N concentrations and significantly differed between the four amino acids tested.

We found a positive effect of soil amino acid concentrations on uptake of mineralized ^{15}N relative to amino acid concentrations for all amino acids which was presumably due to higher diffusion rates of mineralized tracer to the root surface. However, intact amino acid uptake relative to amino acid concentrations as well as the proportion of total ^{15}N taken up directly decreased with increasing soil amino acid N concentrations for all amino acids, irrespective of their microbial degradability. This effect is most likely controlled by the mineral N concentration in soil and perhaps in plants which inhibits direct amino acids uptake.

Overall, we conclude that plant internal regulation of amino acid uptake controlled by mineral N is the main mechanism determining direct uptake of amino acids and thus a lower contribution of intact amino acid uptake to the plants N nutrition has to be expected for higher amino acid concentrations accompanied by mineralization in soil.

Key words:

Microbial competition, organic nitrogen, plant nutrition, stable isotope, ^{15}N, ^{13}C

1 Introduction

It has long been known that plants do have the physiological capacity to take up amino acids in an intact form in sterile hydro solution (Virtanen and Linkola, 1946), but this ability has largely been neglected in natural ecosystems where soil microbes were believed to completely outcompete plants for this nutrient pool (Black, 1993). Investigations studying *in situ* plant uptake of intact amino acids have focused on sub-arctic regions as soil N-pools of these climates are dominated by amino acids (Kielland et al., 2007). However, recent studies have shown that despite of the generally low concentrations of plant available amino acids in soil solutions of temperate climates, the turnover of this N pool is high (Jones and Kielland, 2002; Jones et al., 2004) and might therefore be important also for plants.

Field studies have partly supported this assumption by demonstrating direct plant amino acid uptake in a number of different natural ecosystems, such as Alaskan tussock tundra (Kielland, 1994), boreal coniferous forest (Näsholm et al., 1998), heathland (Stribley and Read, 1980), subtropical rainforest (Schmidt and Stewart, 1999) and even agricultural land (Jones and Darrah, 1994; Yamagata and Ae, 1996; Näsholm et al., 2001). However, studies concerning the ecological relevance of this process are scarce and the importance of direct uptake of organic nitrogen for plant growth is still controversial (Lipson and Näsholm, 2001; Jones et al., 2005a). One major argument against the potential relevance of amino acid uptake by plants in an intact form is the strong competition for amino acids between plants and soil microorganisms (Hodge et al., 2000a; Jones et al., 2005b). The competitive balance between plants and soil microorganisms seems to shift one way or the other depending on abiotic soil conditions like temperature, moisture and inorganic N content (Vinolas et al., 2001b; Bardgett et al., 2002), however the question which factors dominate plant microorganisms competition is so far not completely understood.

Recently, it has been proposed that the concentration of free amino acids in soil solution and their spatial and temporal heterogeneity might be important factors for the outcome of competition for this N source (Hodge et al., 2000a). Indeed, microbial cell lyses and degradation of plant material in soil can lead to significantly higher concentrations of free soil amino acids in micro patches (Jones et al., 2005a; Jones et al., 2005b).

Direct amino acid uptake is commonly investigated by adding labelled amino acids to the soil and tracing the labelled compounds in the plant material. However, the amount of added tracer widely varies from study to study (Table 1.1). As the effect of

amino acid concentrations on their uptake by plants in natural soils is still unclear, a direct comparison between studies using different tracer amounts or between different study sites with variable amino acid concentrations is still not warranted. Even more so when the widely differing chemical characteristics of amino acids need to be taken into account. So far, studies on the relationship between the concentration of amino acids and their uptake by plants focused on only few amino acids (Vinolas et al., 2001a; Jones et al., 2005b), thereby only considering the effect of C/N ratio, and sorption reactions, but not the effect of water solubility or biodegradability. Thus, little is known about the interaction between chemical characteristics and amino acid concentrations and its effect on the uptake rates by plants.

We conducted a greenhouse experiment using *Anthoxanthum odoratum* as a model plant. We adjusted different soil concentrations by applying a set of dual labelled amino acids widely differing in their C/N-ratios, biodegradability and in transport and sorption characteristics in soils (Table 1.2). As the soil amino acid concentration highly influences the amount of amino acids reaching the root surface via mass flux or diffusion we assumed that: 1) higher tracer concentrations in the soil will increase the uptake of total N, as well as N taken up as intact amino acids, both relative to total applied tracer amounts (further referred to as uptake efficiency). As shown by Vinolas and coworkers (2001a) the absolute microbial degradation of amino acid tracer does not decrease with increasing tracer amounts and thus we expect increased mineral N (N_{min}) concentrations with increasing tracer amounts. It is known, however, that higher soil N_{min} concentrations inhibit plant uptake of amino acids (Persson et al., 2003). Therefore, we further hypothesize that 2) increasing soil amino acid concentrations will reduce the contribution of intact amino acid-N uptake to the plant´s total N-uptake. Moreover we hypothesize that 3) the smallest uptake rates for intact tracer molecules will occur for those amino acids having the highest C/N ratios as they are preferentially mineralized by microorganisms (Lipson et al., 1999a).

Table 1.1: List of studies on direct plant uptake of soil amino acids including the types of amino acids used in the study as well as the applied amount of labelled amino acids.

Amino acid	Amount [µg N * g^{-1} soil][a]	Diff. amounts[b]	Study
Gly, aspartic acid	58	No	Schimel & Chapin (1996)
Glycine	6	No	Lipson & Monson (1998)
Glycine	0.36	No	Näsholm et al. (2000)
Glycine	3.76	No	Streeter et al. (2000)
Glycine	0.63	No	Näsholm et al. (2001)
Glycine, arginine	15.76	No	Ohlund & Näsholm (2001)
Glycine, glutamate, lysine	0.26, 0.63, 1.25, 2.55, 4.95, 12.45, 25.05	Yes	Vinolas et al. (2001b)
Glycine	4.8	No	Weigelt et al. (2003)
Glutamic acid, glycine, leucine	0.38	No	Henry & Jefferies (2003)
Glycine, arginine	1.08	No	Persson et al. (2003)
Glycine	6.89	No	Bardgett et al. (2003)
Glycine, serine, phenylalanine, arginine	10	No	Weigelt et al. (2005)
Glycine	0.00015, 0.0015, 0.015, 1.5, 15	Yes	Jones et al. (2005b)
Glycine, aspartic acid, alanine	2.5 and 11	No	Kielland et al. (2006)
Glycine	3.3	No	Rains & Bledsoe (2007)
Alanine	0.027	No	Hofmockel et al. (2007)
Glycine, phenylalanine, serine	7.5	No	Harrison et al. (2008)
Glycine, valine, tyrosine, lysine	0.025, 0.13, 2.5	Yes	Present study

[a]: Used application amount of amino acid N; if original units were based on area or soil volume, we converted those to µg N g^{-1} soil by using an average application depth of 10 cm and an average soil density of 1.3 g cm^{-3}.

[b]: Shows if the effect of application amount on the amino acid uptake has been investigated.

2 Materials and Methods

2.1 Planting

We collected topsoil (2 – 30 cm) from the field site of the "Jena Experiment" (Roscher et al., 2004), which is located on a flood-plain of the Saale river near Jena, Germany (11° 34' 60" East; 50° 55' 60" North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was highly fertilized during the last 40 years of agricultural utilisation (Roscher et al., 2004) until 2002 when the "Jena Experiment" started. Soil was collected in November 2005, sieved to < 6 mm within two days of collection and approximately 100 g dry weight equivalent field soil was filled into pots (5 cm diameter, 10 cm height) with drainage holes at the bottom.

As the target plant community of the "Jena Experiment" is classified as a semi-natural species-rich mesophilic grassland (Roscher et al., 2004), our investigations focused on the dominant functional group of grasses, where *Anthoxanthum odoratum* (L.) was chosen as model plant in this study. Seeds of *A. odoratum* (Rieger-Hofmann, Germany) were germinated on a 1:1 sand : soil mixture and three seedlings in the two leave stage were transplanted into each pot. Pots were placed in a climatic chamber with a constant air temperature of 18 °C, a constant relative air humidity of 60 % and a day-night-cycle of 14 : 10 h. Pots were relocated randomly once a week for seven weeks before the start of the labelling experiment.

2.2 Labelling

Three different nitrogen concentrations (0.025, 0.13 and 2.5 µg N g^{-1} soil) were adjusted in the potted soil by application of amino acid mixtures. The two lower adjusted nitrogen concentrations represent minimum application amounts needed for substance specific measurements (0.025 µg N g^{-1} soil) which will be increasingly important in future experiments, and bulk measurements (0.13 µg N g^{-1} soil). Both concentrations were defined a priori by means of precision of the measurement and size of standard error in field replicates, which should be small enough to enable detection of significant differences between labelled and background samples. While this was done in a preexperiment (data not shown), the high nitrogen concentration (2.5 µg N g^{-1} soil) represents average application amounts commonly used in past studies on intact amino acid uptake (Table 1.1). All pots were filled with the same soil, hence background amino acid concentrations before tracer application were the same for all treatments. As the different amino acid concentrations of the used tracer solutions was the only factor to alter amino acid concentrations in soil, we refer to the added concentration as soil concentrations, given in µg N g^{-1} dry weight soil, in the following. We used equal N amounts of the four amino acids glycine (gly), tyrosine (tyr), lysine (lys) and valine (val) per concentration level (Spectra Stable Isotopes, Columbia, USA). These amino acids differ in a number of chemical, physical and biochemical characteristics (Table 1.2): C/N ratios range from 2 (gly) to 9 (tyr), sorption reactions in soil differ for the used neutral (gly, val, tyr) and basic (lys) amino acids and biodegradability is lower for tyr (aromatic ring structure) than for the other amino acids. For each solution, only one amino acid was dually labelled (^{13}C and ^{15}N) resulting in 12 different treatments (3 concentrations x 4 amino acids). All solutions were prepared with deionised water. Labelling of the four amino acids was 98 atom% ^{13}C and ^{15}N for all C- and N-atoms, respectively.

Table 1.2: Physical and (bio)-chemical characteristics of the applied amino acids.

Amino acid	C / N ratio	Molecular weight (g mol^{-1})	Isoelectric point	Acidity / Basicity	Aromatic rings
Glycine	2	75.1	5.97	neutral	no
Tyrosine	9	181.2	5.66	neutral	yes
Lysine	3	146.2	9.59	basic	no
Valine	5	117.2	5.96	neutral	no

We injected 4 mL aliquots of the amino acid solutions into each pot using a 2.5 mL luer-lock glass syringe (Microliter, Hamilton, Switzerland) with a side-hole needle (length 5 cm, point style, 23 Gauge), which was pierced into the soil to a depth of 5 cm and slowly withdrawn from the starting depth to the soil surface during injection. The aliquots were divided into four injections of 1 mL per pot to ensure a homogeneous dispersion of the amino acid solution. This resulted in an application amount of 2.5, 13 and 250 µg N per pot (100 g soil dry weight). We labelled three replicate pots per treatment as well as three control pots per treatment using mixtures of all unlabelled amino acids leading to a total number of 45 pots (12 labelling treatments + 3 background treatments = 15 treatments x 3 replicates). The unlabelled controls were randomly placed between the labelled pots to provide background abundance levels for ^{15}N and ^{13}C. This background level included ^{13}C, which might have been re-fixed by photosynthesis via $^{13}CO_2$ originating from mineralised soil amino acids of surrounding labelled pots. All plants were harvested 48 h after labelling in accordance with results from Streeter et al. (2000) showing highest uptake amounts after this time period.

2.3 Sample preparation

48 h after application, shoots were cut off and immediately frozen in liquid N_2. Removal of soil from the roots was done by carefully shaking the complete root system. A small sub-sample of this soil was used for determining the gravimetric water content. Remaining soil was withdrawn by ultrasonification of the roots in deionised water (35 kHz and 320 W) followed by rinsing the roots with 0.5 M calcium chloride solution to remove amino acids adsorbed to the root surface. In a final step, roots were rinsed with deionised water and then frozen in liquid N_2. Fresh soil and frozen plant material was stored at –25 °C until further analyses. All plant samples were freeze-dried and ground to fine powder with a ball mill shortly before isotope analysis.

2.4 Instrumentation

All isotope analyses were conducted on an elemental analyser (Carlo Erba, NC 2500) that was coupled with an Isotope Ratio Mass Spectrometer (IRMS, Delta^{plus}, Thermo, Bremen, Germany) via a Conflo II interface (Thermo). Helium 4.6 (99.996 % purity, Riessner, Germany) was used as carrier gas and for dilution of the sample CO_2 peak. The sample amounts were adjusted for ^{15}N and ^{13}C measurements to a content of 20 µg N and 100 µg C, respectively. Sample C and N was transformed to CO_2 an N_2 using an oxidation reactor set to 1040 °C and a reduction reactor working at 600 °C.

Three pulses of either CO_2 (99.7 % purity, Riessner) or N_2 (99.9990 % purity, Riessner) were discharged as reference gases directly into the IRMS during measurements. Online calculation of isotopic ratios was done by setting the reference gases to values of -42 ‰ and 0 ‰ for CO_2 and N_2, respectively. The values were corrected afterwards by a three point calibration for ^{13}C measurements set up with certified standards: Sucrose (CH_6, IAEA, Vienna, Austria, $\delta^{13}C$ = –10.47 ‰), $CaCO_3$ (NBS 19, Gaithersburg, USA, $\delta^{13}C$ = +1.95 ‰) and Acetanilid (Carlo Erba Instruments, Milan, Italy, $\delta^{13}C$ = -34.09 ‰). All standards were measured repeatedly together with the samples within one measuring sequence. The same was done for recalculation of the $\delta^{15}N$ values with the exception that, due to the wider range of sample values, the three point calibration was extended to five certified standards using two types of $(NH_4)_2SO_4$ (IAEA-N1, $\delta^{15}N$ = 0.36 ‰ and IAEA-N2, $\delta^{15}N$ = 20.3 ‰), KNO_3 (IAEA-N3, $\delta^{15}N$ = 4.7 ‰) and two ^{15}N-enriched forms of $(NH_4)_2SO_4$ (USG-26, IAEA, $\delta^{15}N$ = 53.7 ‰ and USG-32, IAEA, $\delta^{15}N$ = 180 ‰).

2.5 Calculation

All given isotope ratios were calculated as δ‰ values according to Craig (1953) using equation (1) with

$$\delta_{\text{sample}} = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \quad (‰) \qquad (1)$$

with

$$R = \frac{at\ \%\ heavy\ Isotope}{at\ \%\ light\ Isotope} \qquad (2)$$

If the plants take up N from sources of different isotopic composition, the resulting change in the plants $\delta^{15}N$ value follows a two component mixing system according to Gearing (1991) as shown in equation (3),

$$R_{sample} = \frac{A_0 * {}^{15}N_0 + A_T * {}^{15}N_T}{A_0 * {}^{14}N_0 + A_T * {}^{14}N_T} \quad (3)$$

where R_{sample} is the isotope ratio of a sample, $\delta^{15}N_{sample}$ the measured $\delta^{15}N$ value, $R_{standard}$ represents the isotope ratio of the certified standard, A_0 is the amount of plant material and $^{15}N_0$ / $^{14}N_0$ are the ^{15}N and ^{14}N concentrations in this material in mol g^{-1}. A_T gives the total amount of tracer N taken up into the plant and $^{15}N_T$ / $^{14}N_T$ are the ^{15}N and ^{14}N concentrations in this tracer material in mol g^{-1}. After solving equation (3) for A_T (equation (4)) it can be related to the total amount of tracer applied ($A_{applied}$) using equation (5).

$$A_T = \frac{{}^{15}N_0 * A_0 - R_{sample} * {}^{14}N_0 \; A_0}{R_{sample} * {}^{14}N_T - {}^{15}N_T} \quad (4)$$

$$^{15}\mathrm{N}_{\mathrm{total}} \text{ uptake efficiency} = \frac{A_T}{A_{applied}} * 100 \quad (5)$$

where "$^{15}N_{total}$ uptake efficiency" is the proportion of tracer-N taken up into the plant (in percent) based on the total amount of applied tracer-N. Another important variable to calculate is the relative contribution of intact N to the total tracer N taken up ("*Intact of total ^{15}N*"). This is calculated using the ^{15}N / ^{13}C excess ratio in the samples relative to the ^{15}N / ^{13}C ratio of the applied tracer, which was set to 100 % (equation (6)) (Näsholm et al., 1998). From this we calculated the total amount of intact tracer uptake (A_{intact}) using equation (7). We also calculated the proportion of tracer-N taken up in an intact form into the plant (in percent) based on the total amount of tracer-N applied (equation (8)). This will be referred to as "$^{15}N_{intact}$ uptake efficiency" in the following.

$$\text{Intact of total } {}^{15}\mathrm{N} = \frac{\left(\frac{{}^{15}N}{{}^{13}C}\right)_{Sample}}{\left(\frac{{}^{15}N}{{}^{13}C}\right)_{Tracer}} * 100 \quad (6)$$

$$A_{\mathrm{intact}} = A_T * Intact\ of\ total\ {}^{15}N \quad (7)$$

$$^{15}N_{\mathrm{intact}} \text{ uptake efficiency} = \frac{A_{\mathrm{intact}}}{A_{applied}} * 100 \quad (8)$$

2.6 Statistics

Statistical analysis was done using *R 2.6.1 for Windows* (http://r-project.org). An analysis of variance (ANOVA) was used to quantify how single variables contribute to the observed variances of the data. Relative amounts of intact amino acid uptake, either

based on total tracer N uptake or based on total applied tracer N, were used as dependent variables, application amount, and type of amino acid were treated as independent variables. All dependent variables were log-transformed to meet ANOVA assumptions. We found significant changes in soil moisture between treatments and therefore soil moisture was always added first into the model to account for its possible effects on uptake patterns before fitting other factors. We used a Tukey post-hoc test to analyse the effects of single amino acids and amino acid concentrations. To account for the effect of soil water content the post-hoc test was calculated using the residuals of a linear correlation between the dependent variable and soil water content.

3 Results

3.1. Total N and amino acid N uptake

An increase of the adjusted amino acid concentrations led to a significant increase of the absolute total tracer derived N (A_T) and of N taken up in an intact form (A_{intact}, Table 1.3). This was true for all amino acids except for the uptake of N in the form of intact gly between 0.025 and 0.13 µg N g^{-1}soil where no significant differences appeared. However intact N-uptake as percentage of total ^{15}N-uptake did decrease with increasing amino acid concentration for all amino acids except valine.

Table 1.3: Absolute total ^{15}N uptake (µg N), absolute intact ^{15}N uptake (µg N) and the relative contribution of ^{15}N taken up in an intact form to the total ^{15}N uptake (*Intact of total ^{15}N* (%)). Given are means ± 1 standard error for the three applied amino acid concentrations.

Concentration		*Total and intact ^{15}N uptake (µg N)*				
		Glycine	**Tyrosine**	**Lysine**	**Valine**	**Average**
0.025 µg N g^{-1} soil	total	0.63 ± 0.07	0.19 ± 0.03	0.20 ± 0.05	0.27 ± 0.014	0.32 ± 0.07
	intact	0.17 ± 0.05	0.13 ± 0.02	0.14 ± 0.05	0.10 ± 0.05	0.14 ± 0.04
Intact of total ^{15}N (%)		27.0	68.4	70.0	37.0	43.8
0.13 µg N g^{-1} soil	total	4.53 ± 0.31	1.79 ±0.23	1.32 ± 0.12	1.14 ± 0.06	2.20 ±0.18
	intact	0.16 ± 0.07	0.25 ± 0.04	0.47 ± 0.09	0.13 ± 0.01	0.25 ± 0.05
Intact of total ^{15}N (%)		3.5	14.0	35.6	11.4	11.4
2.5 µg N g^{-1} soil	total	108.14 ± 11.32	65.53 ± 6.37	56.63 ± 7.26	47.90 ± 5.50	69.55 ± 7.61
	intact	1.50 ± 0.57	3.62 ± 0.81	0.99 ± 0.29	8.42 ± 1.23	3.63 ± 0.72
Intact of total ^{15}N (%)		1.4	5.5	1.8	17.6	5.2

3.2. Uptake efficiencies of total N and amino acid N uptake

With increasing amino acid concentrations, the percentage of ^{15}N taken up relative to the applied tracer N amount (*$^{15}N_{total}$ uptake efficiency*, Figure 1.1A) increased in average from 13 % to 28 %. Moreover, this $^{15}N_{total}$ uptake efficiency of *A. odoratum* significantly differed between the four amino acids with the type of amino acid explaining the largest part of the variability in the ANOVA (Table 1.4A). Differences in adjusted amino acid concentrations and interactions between both factors were also significant, but explained less variability (Table 1.4A). While differences in *$^{15}N_{total}$ uptake efficiency* were small between lys, tyr and val, for all amino acid concentrations, gly permanently showed the highest uptake rates of all used amino acids (Figure 1.1A). This was most pronounced for the low concentration (0.025 µg N g^{-1} soil) where *$^{15}N_{total}$ uptake efficiency* from gly was 2.3 to 3.2 fold higher than for tyr and val, respectively. With higher concentrations these differences became smaller but were still significant even for the highest concentration (2.5 µg N g^{-1} soil).

The *$^{15}N_{intact}$ uptake efficiency* of *A. odoratum* showed a significant decrease from 5.4 % to 1.5 % in average with increasing amino acid concentration (Figure 1.1B, Table 1.4B). This decrease again differed significantly between amino acids (Table 1.4B) with val being the only amino acid for which $^{15}N_{intact}$ uptake efficiency increased again significantly (from 1 % to 3.4 %) at the highest amino acid concentration (ANOVA, post-hoc Tukey $p < 0.01$). In contrast to *$^{15}N_{total}$ uptake efficiency* no consistent hierarchy in the uptake of single amino acids could be found. For *$^{15}N_{intact}$ uptake efficiency*, the largest part of the variability is explained by differences between the amino acid concentration and the interaction between concentration and type of amino acid, while the differences between single amino acids explained a much smaller but still significant amount of variability (Table 1.4B).

3.3. Amino acid N as proportion of total N uptake

Increasing the amino acid concentration led to significant changes in the composition of *A. odoratum's* total N uptake. The proportion of intact amino acid N uptake relative to the total tracer-derived N (*Intact of total ^{15}N*) significantly decreased with increasing amino acid concentrations and significantly differed between amino acids (Figure 1.1C, Table 1.4C). There was a relative drop of 95, 92, 98 and 53 % based on the value at 0.025 µg N g^{-1} soil for gly, tyr, lys and val, respectively between 0.025 and 2.5 µg N g^{-1} soil. This overall decrease was most pronounced from 0.025 to 0.13 µg N g^{-1} soil with a significant relative drop of 87, 79, 47 and 68 % for gly, tyr, lys and val,

respectively (Figure 1.1C). Only for lysine *Intact of total ^{15}N* also decreased significantly by 36 % from 0.13 to 2.5 µg N g^{-1} soil (ANOVA, post-hoc Tukey $p < 0.01$, Figure 1.1C). Thus, *Intact of total ^{15}N* significantly depended on the type of amino acid and on the amino acid concentrations as also indicated by the significant interaction between both terms (Table 1.4C).

Table 1.4: Analysis of variance of the effect of soil amino acid concentration and type of amino acid on the percentage of ^{15}N (A) taken up relative to the applied tracer N amount (^{15}N total uptake efficiency), (B) taken up in an intact form (^{15}N intact uptake efficiency) and (C) the relative contribution of ^{15}N taken up in an intact form to the total ^{15}N uptake (Intact of total ^{15}N).

Source	d.f.	SS	%SS	MS	F	p
A $^{15}N_{total}$ uptake efficiency						
Soil water content	1	2.60	23.0	2.60	47.38	<0.001
Type of amino acid (AA)	3	5.06	44.7	1.69	30.73	<0.001
Concentration	2	2.00	17.7	1.00	18.18	<0.001
AA x Concentration	6	1.10	9.7	0.18	3.34	0.045
Residuals	10	0.55	4.9	0.05		
B) $^{15}N_{intact}$ uptake efficiency						
Soil water content	1	10.00	20.2	10.00	37.65	<0.001
Type of amino acid (AA)	3	10.30	20.8	3.43	12.93	<0.001
Concentration	2	14.72	29.8	7.36	27.73	<0.001
AA x Concentration	6	11.76	23.8	1.96	7.38	0.003
Residuals	10	2.65	5.4	0.27		
C) Intact of total ^{15}N						
Soil water content	1	3.16	15.5	3.16	23.61	<0.001
Type of amino acid (AA)	3	1.96	9.6	0.65	4.89	0.024
Concentration	2	6.33	31.1	3.17	23.69	<0.001
AA x Concentration	6	7.56	37.2	1.26	9.43	0.001
Residuals	10	1.34	6.6	0.13		

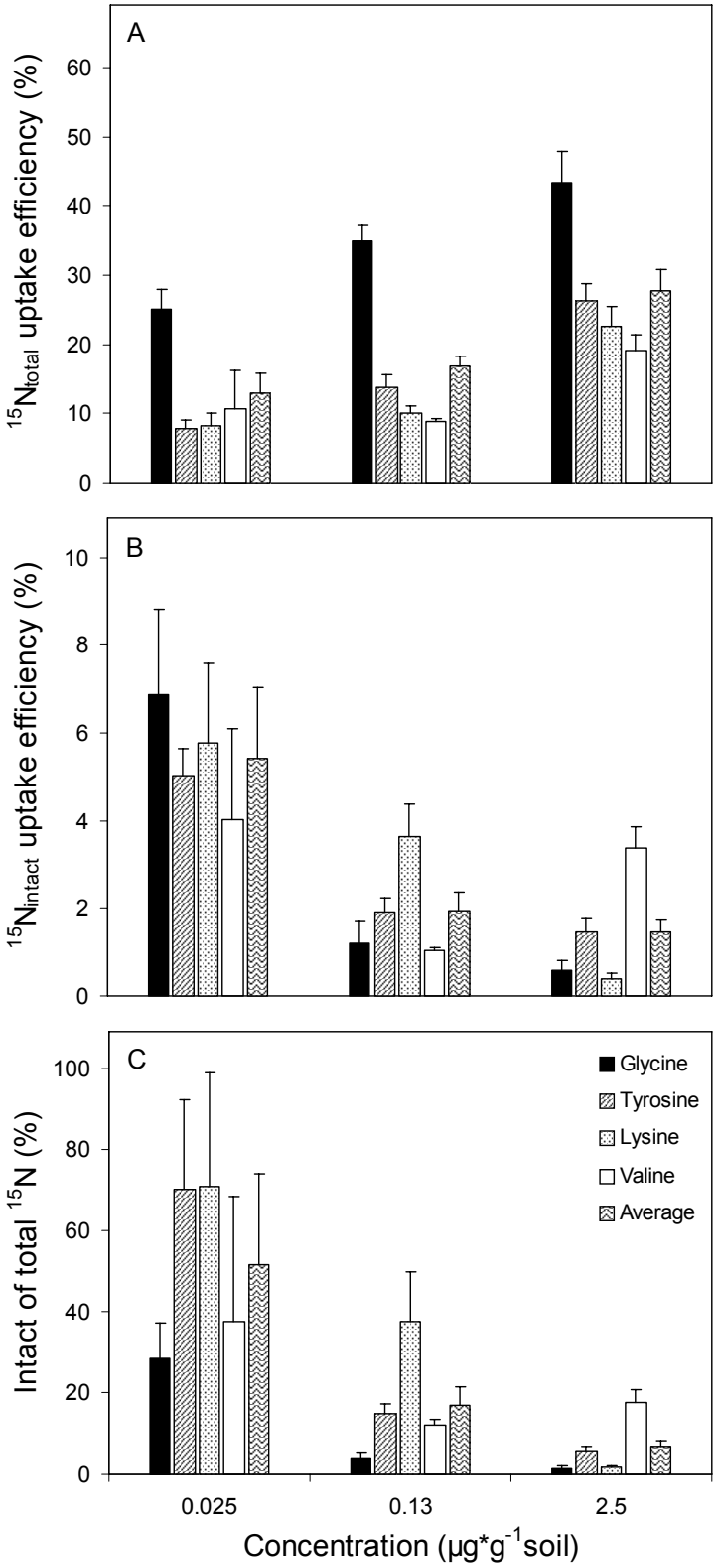

Figure 1.1: ^{15}N uptake of four different amino acids for three different soil amino acid concentrations depicted as: (A) total ^{15}N uptake for the whole plant (*$^{15}N_{total}$ uptake efficiency*) and (B) uptake of intact ^{15}N (*$^{15}N_{intact}$ uptake efficiency*) each as proportion of the applied tracer amount and (C) contribution of intact N uptake to the total N uptake (*Intact of total ^{15}N*); bars show mean values ± standard error.

4 Discussion

4.1 Abiotic mechanisms controlling total ^{15}N uptake and direct amino acid uptake

We hypothesized that increasing amino acid concentrations would lead to higher uptake efficiencies of amino acids and amino acid-derived N. Our data supported this hypothesis for the $^{15}N_{total}$ uptake efficiency, which significantly increased with increasing amino acid concentration in the soil (Table 1.4A). However, although the absolute $^{15}N_{intact}$ uptake increased (Table 1.3), the *$^{15}N_{intact}$ uptake efficiency* (Figure 1.1B) significantly decreased with increasing soil amino acid concentration. Thus the first hypothesis was only partly supported if uptake of intact amino acids is taken into account. Overall, higher amino acid concentrations in the soil resulted in a higher uptake of tracer-derived mineral N but to a proportionally lower uptake of intact amino acids.

According to Fick´s 2nd diffusion law, a higher concentration gradient between the root surface and the surrounding soil in an unsteady diffusion system would lead to an accelerated compensation of concentration differences in time and thus to a disproportional higher transport of tracer to the root surface. This would not only lead to a higher absolute amount of transported tracer but also to a higher relative portion of tracer reaching the root surface. Our data suggests that this elevated diffusive transport of tracer-derived mineral N to the roots is the main driver for the increased mineral N uptake, both absolute and relative to the applied amount, with increasing amino acid concentration. Alternatively, the competitive balance for mineral N uptake between plants and microorganisms might change with changing substrate concentrations, but Hodge et al. (2000a) found evidence for the contrary. They showed that plants do not compete more effectively for mineral N than microorganisms, not even in micro patches of organic material with high N_{min} concentrations (Hodge et al., 2000). However, the diffusion law should equally apply to the transport of intact amino acids leading to a higher uptake efficiency for intact amino acids. Indeed other studies found a positive correlation between uptake efficiencies of intact amino acids and amino acid concentration in soil (Jones et al., 2005b). However the authors suggested no physical relationships being responsible for this effect but only biotic influences. As the progress of intact amino acid uptake (*$^{15}N_{intact}$ uptake efficiency*) in our study does not follow that of *$^{15}N_{total}$ uptake efficiency*, which we suggested to be mainly driven by physical powers, our data also suggests that other than pure physical relationships must play a role in direct amino acid uptake. This could for example be altered uptake capacities of plants

as proposed by Persson et al. (2002; 2003) and thus is directly related to our second hypothesis which will be discussed in detail in the next paragraph.

4.2 Biotic mechanisms regulating direct amino acid uptake

We did hypothesize that an increase in soil amino acid concentrations would lead to a reduced relative contribution of amino acid uptake to the plant's total N uptake. Our results confirmed this second hypothesis, since they show a significant decrease in *Intact of total* ^{15}N with increasing amino acid concentration for all amino acids (Figure 1.1C), except for lys whose different pattern will be discussed in the next section. However, two fundamental processes might explain the general negative relationship between amino acid concentration and *Intact of total* ^{15}N. First, microbes might become more competitive at higher amino acid concentrations in soil and a smaller portion of this N source would therefore be plant available. Second, plants might down-regulate their amino acid uptake, which is known to be an active transport depending on the plants' N-nutrition status (Liu and Bush, 2006). This transport is regulated transcriptionally and post-transcriptionally and might change with increasing tracer amounts.

A higher competitive power of microbes at higher amino acid concentrations might result in an amino acid uptake hierarchy where easily decomposed amino acids are less important for plant uptake compared to slower degrading ones. However, we found no hierarchical uptake pattern for the used amino acids in our data being consistent for all used tracer amounts, which would support this theory. Thus it seems that the plants internal regulation of its N supply plays the dominant role in controlling amino acid uptake. Persson et al. (2002; 2003) showed that *Pinus sylvestris* seedlings revealed smaller absolute uptake rates for amino acids when pre-treated with NH_4^+. The microbial decay of amino acids in our experiment will equally lead to a release of inorganic nitrogen into the soil solution and could have the same effect as a pre-treatment with NH_4^+. The authors assumed that NH_4^+ acts as a key substance for the down-regulation of amino acid transporters (Persson et al. 2002, 2003b). Our findings are consistent with this theoretical model and are supported by the observed increase in total N uptake for higher amino acid concentrations. This implies that soil N_{min} concentrations predominate the regulation of direct amino acid uptake relative to the total N supply. At the same time the positive effects of higher diffusion fluxes seem to play a minor role. As no significant change for the intact uptake efficiency could be detected for high tracer concentrations in our study, we suggest that the plant internal control mechanism is most effective at low to medium soil concentrations. For amino acid concentrations be-

ing at levels far above natural soil concentrations, passive processes i.e. higher diffusive transport, seem to counterbalance this active plant control mechanism in our investigation leading to a constant intact uptake efficiency.

4.3 Effect of different amino acids and their concentration

We deliberately used four amino acids differing in their (bio-)chemical and physical characteristics such as C/N ratio, sorption properties and biodegradability. We hypothesized that, amino acids possessing aromatic ring structures or having low C/N ratios are more difficult or less attractive to degrade for microbes than other amino acids, resulting in high soil amino acid availabilities and higher uptake rates by plants. This hypothesis is contrasting the fact, that most organic substance classes found in soil are known to have a higher degradability with decreasing C/N ratio, due to N limitation of microbes (Scheffer and Schachtschabel, 2002). However, our assumption on biodegradability is supported by a number of studies (Alef and Kleiner, 1986; Kielland, 1995; Lipson et al., 1999a) which found low mineralization rates in soil, especially for gly and glutamate (glu). According to these authors, their findings are due to the extremely low C/N ratios of the investigated amino acids, i.e. then being lower than the average ratio of microbial biomass. Hence degradation of these molecules is rather C than N limited. Our results, however, were not consistent with this simple classification and rather indicate a complex interplay of concentration and amino acid type effects.

As the plant's physiological regulation of amino acid uptake is the dominant driving force, the intensity of the observed trends should be the same for all amino acids as mixtures of all four amino acids had been applied in each treatment. Indeed, uptake patterns of all amino acids decreased (for $^{15}N_{intact}$ *uptake efficiency*) or increased (for $^{15}N_{total}$ *uptake efficiency*) with increasing amino acid concentrations suggesting a tight co-regulation of different amino acid transporters as it was also found by Persson et al. (2003). The amount of uptake significantly differed between amino acids as did the interaction with amino acid concentration (Table 1.4 B, C), but the uptake hierarchy cannot be explained easily with single mechanisms: Gly and tyr are potentially poor microbial substrates either due to the low C/N ratio (Lipson et al., 1999b) or due to the aromatic ring structure, which should maximise their availability to plants. However, tyr does not significantly differ from the other amino acids. In contrast, gly is clearly the least important amino acid for direct uptake. Obviously, gly undergoes rapid microbial decay irrespective of its low C/N ratio, probably due to co-metabolic degradation of carbon rich soil organic matter. This decay results in a high N release because microbial

biomass has an average C/N ratio of about 5.7 in grassland (Scheffer and Schachtschabel, 2002). Consequently, gly derived N shows the highest $^{15}N_{total}$ uptake efficiency. At the same time val, which has the highest C/N ratio and should represent a favourable source for microbes, revealed higher values for *Intact of total ^{15}N* than i.e. gly.

Finally, lys differed from all other amino acids revealing a strong and significant decrease (ANOVA, post-hoc Tukey $p < 0.01$) of *Intact of total ^{15}N* between intermediate (0.13 µg N g^{-1} soil) and high (2.5 µg N g^{-1} soil) amino acid concentration (Figure 1.1C). Lys is a basic amino acid and its positive charge will lead to increased sorption reactions in soil compared to the applied neutral amino acids and therefore should exhibit a lower availability for microbial decay (Jones and Hodge, 1999). When higher amounts of lys are applied, more of the sorption capacity of the soil matrix will be occupied and the relative availability of lys for the microbial population will increase. In this situation, microbial low affinity but high capacity enzymes might out compete plant high affinity transporters for lys in the root (Fischer et al., 1998) thereby lowering the availability of lys for plants dramatically. Because of the following microbial breakdown of lys the additional release of N_{min} into the soil solution will increase the N_{min} supply of the plants and plant internal uptake regulation will down regulate lys uptake, leading to lower lys uptake at high amino acid concentrations.

Moreover, we found no significant changes in the uptake of intact amino acids (*$^{15}N_{intact}$ uptake efficiency* and *Intact of total ^{15}N*) from intermediate to high amino acid concentration. Jones et al. (2005b) suggested that the capacity of the microbial community to take up amino acids becomes saturated at high exogenous concentrations (> 15 µg N g^{-1} soil) which indeed would enhance amino acid availability for plants, therefore balancing the negative effect of plant internal uptake control mechanisms. This actually could explain the trend of *Intact of total ^{15}N* in our study from medium to high concentration. However, as already mentioned in the preceding paragraph any shift in the plant-microbial competition with amino acid concentrations is unlikely and might only appear for very high concentrations as used by Jones et al. (2005b). A diminished microbial mineralization rate on the other hand would also reduce the effect of plant internal uptake regulation for intact amino acids as it would lower the availability of mineral N being the signal substance for this regulation mechanism. Neither our data on the absolute total N uptake (Table 1.3) support this hypothesis nor the investigations of Vinolas et al. (2001a) who found no saturation of the microbial potential for amino acid mineralization at concentrations used in our study. We therefore suggest that plant in-

ternal down-regulation of the activity of amino acid transporters is already at its maximum for the medium concentration level and an additional increase of amino acid concentrations has no further effect on the uptake efficiency for intact amino acids.

5 Conclusions

We were able to show that intact uptake of amino acid N and uptake of total amino acid derived N was clearly influenced by concentration changes of amino acids in soil.

The negative effect of higher soil amino acid concentrations on the plants' uptake efficiency for intact amino acids was shown to be independent of the structure of the amino acids, and their microbial degradability. Therefore plant to microbe competition seems to be insufficient to explain the plants' uptake dynamics for intact amino acids in our study.

The contrasting increase of uptake efficiency for total tracer derived N with rising amino acid concentration suggests, that plant internal down regulation of amino acid transporters at a high internal N status is a more feasible mechanism to explain these trends.

On the ecologic level this implies, that a lower contribution of intact amino acid uptake to the plant's N nutrition has to be expected for higher amino acid concentrations in soil. As a methodological consequence comparisons between studies using different tracer amounts or being conducted on sites with different soil amino acid concentrations can only be done when this effect is taken into account. In the future more effort has to be made to lower tracer amounts and therefore minimize the disturbance of the investigated systems assuring a high accuracy of the calculated amino acid uptake.

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Study 2: Advantages of compound-specific stable isotope measurements over bulk measurements in studies on plant uptake of intact amino acids

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Abstract

Increasing interest in the ability of plants to take up amino acids has risen questions on the accuracy of the commonly used bulk method to measure and calculate amino acid uptake. While this method uses bulk measurements of ^{13}C and ^{15}N enrichment in plant tissues after application of dual labelled amino acids, some authors have recommended the use of compound-specific stable isotope (CSI) analysis of the plants' amino acids instead. However, there has never been a direct evaluation of both methods so far.

We conducted a field study applying dual labelled (^{13}C, ^{15}N) amino acids (glycine, valine, tyrosine and lysine) to soil of a *Plantago lanceolata* monoculture. Root and shoot samples have been collected 24 h after label application and the isotope composition of plant tissues was investigated using bulk and CSI measurements.

Enrichment of ^{13}C in the case of CSI measurements was limited to the applied amino acids showing that no additional ^{13}C has been incorporated into the plants' amino acid pool via the uptake of tracer-derived C-fragments. Compared to this rather conservative indicator of amino acid uptake, ^{13}C enrichment of bulk measurements was 8, 5, 1.6 and 6 times higher for fine roots, storage roots, shoot and the whole plant, respectively.

These findings show that the additional uptake of tracer derived C-fragments will result in a considerable overestimation of amino acid uptake in the case of bulk measurements. We therefore highly recommend the use of CSI measurements for future amino acid uptake studies due to their higher accuracy.

Key words: amino acid uptake, compound specific, stable isotopes, ^{15}N, ^{13}C

1 Introduction

Plants have the physiological capacity to take up amino acids in an intact form, not only in sterile hydroponics (Virtanen and Linkola, 1946), but also in natural soils. This has first been studied *in situ* in soils of the sub-arctic region (Kielland, 1994), but has meanwhile been demonstrated for soils of boreal (Näsholm et al., 1998) temperate (Stribley and Read, 1980) and sub-tropic (Schmidt and Stewart, 1999) climates and even in agricultural systems (Näsholm et al., 2000). A rising number of studies demonstrates the growing scientific interest in the use and possible importance of direct organic nitrogen uptake as alternative organic N source for the plants. Amino acids might significantly contribute to the total plant N uptake, especially in poor soils with a small mineral N pool where this potential might have large effects on nutrient cycling (Kielland, 1994). Moreover, different plant species take up different amounts of amino acids, depending on their growth strategies (Harrison et al., 2008) or habitat types (Weigelt et al., 2003), which might facilitate nutrient partitioning and species coexistence. However, the correct measurement of direct amino acid uptake is the key to our understanding of such processes.

Dual stable isotope labelling (^{15}N, ^{13}C) is the most frequently used method to measure intact amino acid uptake. Labelled amino acid solution is injected into the soil and isotope enrichment of ^{15}N and ^{13}C is thereafter measured in bulk plant material. According to Näsholm et al. (1998) a significant linear correlation between ^{13}C and ^{15}N enrichment in plant samples proofs direct uptake of intact amino acids. The proportion of N taken up in an intact form is calculated by comparing the measured ratio of ^{13}C to ^{15}N excess in plant material to the theoretical ^{13}C:^{15}N ratio of the tracer molecule which is set to 100%. This technique allows the differentiation between N uptake from unlabelled soil N pools and simultaneous uptake of labelled amino acid-derived N. Despite some flaws, the method published by Näsholm et al. (1998) is most frequently applied to calculate intact amino acid uptake from this kind of data. The problems with this method are mainly due to the assumption that any ^{13}C enrichment found in plant material is caused by the uptake of ^{13}C as intact amino acid tracer molecule. According to this assumption, changes in the plant's ^{13}C:^{15}N enrichment ratio could only be caused by changes in intact uptake of ^{13}C or by changes in uptake of mineral ^{15}N derived from microbial tracer decay in soil. As a result, the maximum ^{13}C:^{15}N enrichment ratio in plants would be the ratio of the used tracer itself (Figure 2.1 A). However, some results in literature question this assumption e.g. that uptake calculations of tracer-derived N in

form of intact amino acid uptake sometimes results in maximum values of more than 100 % uptake (Nordin et al., 2001; Weigelt et al., 2003), and thus the enrichment ratio in the plant was higher than that of the used amino acid tracer. This overestimation could be the result of two processes: First, labelled amino acids are subject to microbial cleavage, producing labelled C and N fragments in the soil. Plant uptake of these C skeletons would lead to an overestimation of intact uptake (Näsholm and Persson, 2001) (Figure 2.1 B). Second, plant internal transformation and degradation of the taken up amino acids. A desamination of amino acids in the root followed by the transport of released $[^{15}NH_4]^+$ to the shoot would also lead to a relative enrichment of ^{13}C in the root and therefore overestimate intact amino acid uptake in root material (Figure 2.1 C). For glycine, there is yet a third mechanism through microbial metabolism via the glycine decarboxylase pathway. Here, two molecules of glycine form one molecule of serine together with ammonia and carbon (Oliver, 1994). If this happens to two labelled glycine molecules and the produced serine is then taken up by plants, the resulting ^{13}C:^{15}N enrichment ratio of plant material will be 3 : 1 instead of 2 : 1 for glycine which will again result in an overestimation of intact uptake (Figure 2.1 E). There is also the possibility of underestimating intact amino acid uptake via decarboxylation of tracer amino acids in the plant and subsequent loss of $^{13}CO_2$ (Figure 2.1 D). However, this can and is mostly controlled for through proper experimental design. The problem with using bulk measurements is that we cannot differentiate between ^{13}C uptake in the form of the applied amino acids and uptake of other ^{13}C enriched molecules derived from the microbial breakdown of tracer in soil. The different pathways illustrated in figure 2.1 thus cannot be separated via this method nor can we assess their importance.

A more general methodological constraint using stable isotope technique is the detection of ^{13}C enrichment in plant material. This can be especially difficult when low levels of amino acid tracer are used in order to avoid alteration of the size of the natural amino acid pool in soil. Several studies found considerable amounts of ^{15}N in plant shoots while significant ^{13}C enrichment was not detected (Lipson and Monson, 1998; Hodge et al., 1999; Hodge et al., 2000b). Apart from decarboxylation processes of amino acids in the plant root this mismatch is mainly related to a stronger dilution of the ^{13}C label compared to ^{15}N. On the one hand, plant C content is much higher than plant N content (45 – 50 % C compared to 3 – 5 % N in the dry weight) and on the other hand the natural ^{13}C content of plants is higher (ca. 1.08 % for C3 plants) compared to ^{15}N,

leading to a dilution of ^{13}C that is 60 – 150 times higher than that of ^{15}N (Näsholm and Persson, 2001).

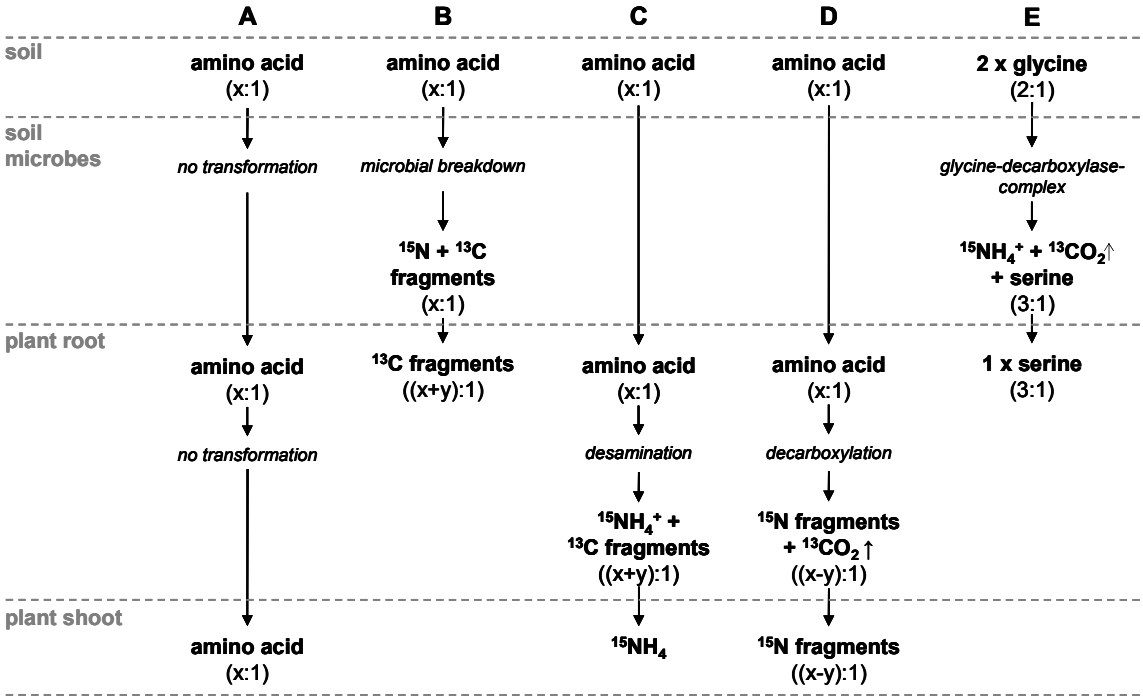

Figure 2.1: Depiction of possible pathways for amino acid uptake into plants. Numbers in brackets give the expected ^{13}C : ^{15}N enrichment ratios in soil, soil microbes, plant roots and shoots where x stands for the number of C-atoms in an amino acid and y stands for the number of C atoms present in the form of C-fragments.

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

Despite the criticism of the Näsholm calculation method using bulk measurements (Persson and Näsholm, 2001) and the suggestions to use the more sensitive CSI method (Nordin et al., 2001), there is yet no consistent comparison of both methods.

Therefore, the goal of this study was to evaluate both methods in a field experiment using four types of dual labelled amino acids.

2 Material and Methods

2.1 Field site

In April 2006 we established a monoculture of *Plantago lanceolata* by sowing and planting of seedlings on the Jena Experiment field site (Roscher et al., 2004). Seedlings were grown in pots for 8 weeks and transferred to the field simultaneously with the sowing of seeds (Rieger-Hofmann, Blaufelden-Raboldshausen, Germany). The field site is located on a flood-plain of the Saale river near Jena, Germany (11°34'60" East; 50°55'60" North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was highly fertilized during the last 40 years of agricultural utilisation until 2002 when the *Jena Experiment* started. In August 2006 undisturbed soil cores from the monoculture plot were collected by pressing a poly ethylene (PE) tube (diameter 30 cm, height 30 cm) pneumatically into the soil. The base area of the soil cores was straightened by breaking off soil pieces using a spatula. This was done to avoid the sealing of macro pores with soil as a consequence of simply slicing the overlaying soil with a knife. Five soil cores were collected and brought to a 35 cm deep trench. The soil cores were placed upright in the trench leaving 5 cm of free space at the bottom to allow free drainage. After the installation the lateral free space between soil cores and the trench was filled with field soil to allow for natural soil temperature dynamics. As soil respiration might have been influenced by the cutting of roots when pressing the PE tube into the soil, the cores were allowed to equilibrate for one week before the labelling started.

2.2 Labelling

A mixture of four dual labelled (^{13}C and ^{15}N, Spectra Stable Isotopes, Columbia, USA) amino acids (glycine, valine, tyrosine, lysine) was applied to four soil cores. Enrichment of the uniformly labelled amino acids was 98 at % both for ^{13}C and ^{15}N. The fifth soil core received a mixture of the four unlabelled amino acids and was used to measure natural isotope abundance values. We injected 34 mL aliquots of the tracer solution into each core using a 2.5 mL luer-lock glass syringe (Microliter, Hamilton, Bonaduz, Switzerland) with a side-hole needle (length 5 cm, point style, 23 Gauge), which was pierced into the soil to a depth of 5 cm and slowly withdrawn during injec-

tion. The aliquots were divided into 17 injections of 2 mL per core that were arranged circular around the centre to ensure a homogeneous dispersion of the amino acid solution. This led to a total application amount of approximately 0.06 mmol of each of the used amino acids.

2.3 Sampling and sample preparation

Aboveground biomass of the four labelled soil cores and the unlabelled core was cut 24 h after application and immediately frozen in liquid N_2. The PE tubes containing the soil cores were cut open using a small circular saw and three 2 cm soil slices were cut from the centre to the edge of the core, serving as soil subsamples for all further soil analysis. The rest of the soil core was dried at 55 °C for seven days to measure the dry weight. An aliquot of the soil sub-sample was used to determine the gravimetric water content of the soil. Roots were extracted as outlined in Sauheitl et al. (2009). After a second rinse with deionised water, roots were separated into fine roots (< 2 mm) and storage roots (>2 mm), frozen in liquid N_2 and stored at -40 °C until further analysis. All plant material was freeze-dried and ground to fine powder with a ball mill shortly before amino acid extraction and bulk measurements were performed. The sampling resulted in a total of four replicates for the aboveground biomass and the two types of roots for the labelled soil cores, respectively, plus the samples from the unlabelled core.

2.4 Amino acid extraction, purification and derivatization

Amino acid extraction of the plant material was done using hot acidic hydrolysis which ensured the extraction of free and protein-bound amino acid molecules. The hydrolysis, purification and derivatization was done according to Amelung and Zhang (2001) with some modifications: In brief 15 mg of shoot or 30 mg of root material were spiked with 35 µg Norvaline (Sigma Aldrich, Seelze, Germany) as first internal standard (IS1) and then hydrolysed. After purification the liquid samples were freeze-dried and derivatized. Volumes of used derivatives were changed compared to Amelung and Zang (2001) to 500 µL water free 4 M HCl and 150 µl pentafluorpropionic acid anhydride. Before derivatization, 35 µg of trans-4-(aminomethyl) cyclohexane carboxylic acid (Sigma Aldrich) were spiked to each sample as second internal standard (IS2). The resulting solution was then transferred to gaschromatograph (GC) vials and measured on an isotope mass spectrometer (see below). Parallel to the samples a mixture of 12 amino acids at a concentration range of 13 – 106 µg mL^{-1} was derivatized serving as

reference standards for quantification and corrections of delta values as described in detail below.

2.5 Measurement

All compound-specific isotope measurements of ^{13}C and ^{15}N were performed on an isotope ratio mass spectrometer (Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany) which was coupled with a gas chromatograph (GC, Trace GC 2000, Thermo Finnigan) via a combustion interface (GC Combustion III, Thermo Finnigan). Except for GC settings, detailed instrument setup and info on the referencing procedure during measurement can be found in Sauheitl et al. (2005). 2 µL of sample solution were injected into the GC with an autosampler (AS 2000, Thermo Finnigan) working with a 10 µL syringe with 70 mm needle length (Hamilton, Switzerland). Evaporation of the samples in the GC was done at 250 °C injector temperature in a glass liner that had been deactivated in 5 % dimethylchlorsilane in toluene for at least one week. A BPX5 column (60 m long x 0.25 mm i.d. x 0.25 µm film thickness of a cross linked polymer of 5 % diphenyl- and 95 % polysiloxane) was used for separation of the single amino acids. The helium (99.996 % purity) flow through the column was kept constant at 1.1 mL min^{-1} and starting temperature of the GC oven was 80 °C held for one minute then raised to 140 °C with a rate of 2.0 °C min^{-1}, raised to 200 °C with a rate of 10 C min^{-1}, raised to 220 °C with a rate of 15 °C min^{-1} held for two minutes, raised to 240 °C with a rate of 20 °C min^{-1} and held for 5 minutes, and finally raised to 300 °C at a rate of 60 °C min^{-1} and held for another 5 minutes. Each sample was measured in four-fold replication and ^{13}C and ^{15}N measurements were performed in subsequent runs in which the same GC settings were used.

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

Online calibration of delta values was done using CO_2 and N_2 reference gases as generally described in Glaser et al. (2002). For correction of derivatization C in the amino acid derivates, delta values of all investigated amino acids were also measured in the underivatizised amino acid standards using an elemental analyser (EA, Carlo Erba NC 2500, Thermo Finnigan) which was coupled with the IRMS (EA-IRMS) via a Con-

flo III interface (Thermo Finnigan) instead of the GC. Details on the used EA calibration are given in Sauheitl et al. (2009b).

2.6 Calculations

Isotope measurements where performed using the δ notation as output. For all following calculations this was transferred to at % heavy isotope according to Craig et al. (1953).

As for CSI measurements amino acids have to be derivatizised beforehand, the measured at% ^{13}C values include the added derivatization C. This will change the original ^{13}C signal of the pure amino acid according to equation (1).

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

With *Z* representing the number of C atoms in the derivative (*derivative*) and the pure amino acid (*AA*) and showing the number of C atoms transferred during derivatization in the form of pentafluorpropionic anhydride (*PFPA*) and isopropanol (*Isop*). *At%* gives the isotopic composition of each substance which in the case of amino acid, and derivatization reagent was measured using the EA-IRMS. Glaser and Amelung (2002) were able to remove this derivatization effect using equation (2):

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

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

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

$$at\%_{AA,derivative,s} = at\%_{AA,derivative,measured} - \left(t_{AA,derivative} * \frac{t_{refgas2} - t_{refgas1}}{at\%_{refgas2} - at\%_{refgas1}} \right) \quad (3)$$

In this equation the measured isotope composition of an amino acid derivative in a sample or standard ($at\%_{AA,\,derivative}$) is corrected for the drift of at% values between the first reference gas ($at\%_{refgas1}$) in the chromatogram and the first reference gas after the amino acid derivative peak ($at\%_{refgas2}$), at which *t* is the retention time of the amino acid derivative or the reference gas.

When plants take up ^{13}C- and ^{15}N-enriched material, this will mix with the already existing plant C and N pool. The resulting new isotope composition of the plant material can be calculated according to Gearing (1991) via a two component mixing system (equation (4)).

$$R_{sample} = \frac{A_0 * \text{heavy isotope}_0 + A_T * \text{heavy isotope}_T}{A_0 * \text{light isotope}_0 + A_T * \text{light isotope}_T} \quad (4)$$

With A_0 representing the amount of plant C or N in mol and *heavy / light isotope$_0$* giving its concentration of heavy and light isotope before tracer application in at%. A_T gives the amount of tracer C or N taken up into the plant and its isotope composition of heavy and light isotopes both in at %.

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

$$A_T = \frac{\text{heavy isotope}_0 * A_0 - R_{sample} * \text{light isotope}_0 \; A_0}{R_{sample} * \text{light isotope}_T - \text{heavy isotope}_T} \quad (5)$$

We further refer to A_T as *^{13}C or ^{15}N enrichment or excess*. In the case of compound-specific measurements, this was separately done for each of the four used amino acids. Total plant heavy isotope excess was calculated as sum of enrichments found in the single amino acids and plant compartments. For both methods, the assessed enrichments per plant tissue were expressed relative to total uptake per plant and referred to as *proportion of total uptake* (6).

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

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

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

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

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

2.7 Statistics

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

3 Results

3.1 Comparison of bulk and CSI measurements

Correlations between ^{15}N and ^{13}C enrichment given as excess in µmol in root and shoot samples were highly significant ($p < 0.01$) with an R^2 of 0.943 and 0.999 for the fitted linear regressions for root and shoot samples, respectively. The enrichment of ^{13}C was significantly positive ($p < 0.05$) for all investigated plant tissues and for both methods. Both methods showed a declining trend of ^{13}C uptake from fine roots to shoots to storage roots (Figure 2.2). We found significantly ($p < 0.05$) different ^{13}C uptake rates between the two methods in single plant compartments and the whole plant material with bulk measurements resulting in a 8, 5, 1.6 and 6 fold higher ^{13}C enrichment compared to CSI measurements for fine roots, storage roots, shoots and the whole plant, respectively. The relatively low allocation of ^{13}C to the shoot in the case of bulk measurements resulted in the lowest ^{13}C : ^{15}N ratio of all plant tissues (Table 2.1).

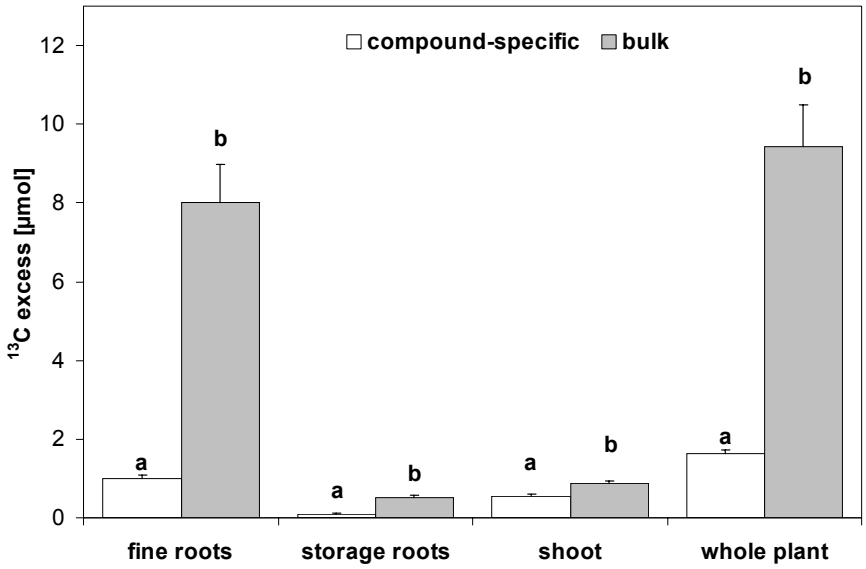

Figure 2.2: ^{13}C enrichment in three plant tissues and the whole plant measured via bulk (grey bars) or CSI measurement (open bars) of the target amino acids. Bars show mean values ± standard error; different lower case letters show significant differences between both measurements for one plant tissue ($p<0.05$).

Table 2.1: ^{13}C and ^{15}N excess plus ^{13}C : ^{15}N ratio for single and all (CSI total) target amino acids and in bulk samples as derived from the CSI and bulk measurements in three different plant tissues and the whole plant. Significant differences are indicated by different lower case letters as derived from post-hoc tests ($p < 0.05$).

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

[1]: Significant differences (ANOVA) between amino acids or types of measurement within a compartment.

[2]: Significant differences (ANOVA) for one specific amino acid or measurement between different compartments.

[3]: ANOVA not conducted as 'whole plant' is not a specific plant compartment.

3.2 Isotope enrichment in single amino acids

CSI measurements showed that tracer C and N uptake into different plant compartments differed among individual amino acids (Table 2.1, Figure 2.3A). Though peak separation between tyrosine and lysine was not complete (Figure 2.4), sensitivity analysis showed that the position of the parting line between both peaks did not influence the overall enrichment of both peaks significantly. Even though this poor peak separation did therefore not affect the outcome of our method comparison, we will only give the combined enrichment of both amino acids in all figures and tables, as this reflects the chromatographic potential of our method. Highest relative ^{13}C uptake was found in fine roots (61 %), followed by shoots (33 %) and storage roots (6 %). In fine roots, tyrosine and lysine showed the highest relative uptake (30 %) followed by valine (20 %) and significantly less uptake of glycine (11 %). This pattern was also found for storage roots with 2.6, 1.9 and 1.1 % relative uptake for tyrosine/lysine, valine and glycine, respectively. In shoots relative uptake of valine (17 %) was highest followed by tyrosine/lysine (11 %) and again significantly less uptake of glycine (6 %) (Figure 2.4A). Relative uptake of ^{15}N, however, shows a different picture (Table 2.1, Figure 2.4B): Most tracer-derived ^{15}N that was coupled to the investigated amino acids was found in shoot material (64 %) followed by fine roots (33 %) and storage roots (3 %). The highest portion of this overall uptake was bound to glycine (42 %), while tyrosine/lysine and valine only accounted for 30 and 28 %, respectively. The same distribution was found for fine roots and shoot material whereas relative ^{15}N uptake of single amino acids was not different in storage roots. The ratio of ^{13}C : ^{15}N enrichment in all plant tissues and for all applied amino acids was lower than that of the original tracer molecules. This ratio differed between amino acids and was significantly smaller in shoot than in root tissues (Table 2.1).

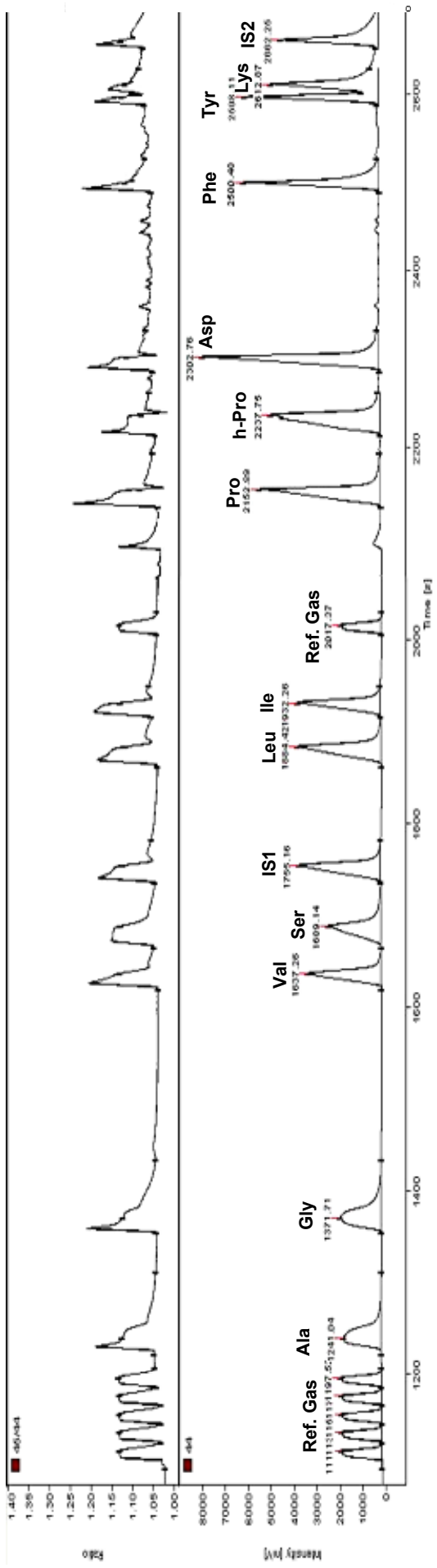

Figure 2.3: Chromatogram of an amino acid standard. Peaks give signal intensity [mV] of CO_2 derived from oxidation of alanine (Ala), glycine (Gly),, valine (Val), serine (Ser), first internal standard (IS1) leucine (Leu), isoleucine (Ile), proline (Pro), hydroxy-proline (h-Pro), aspartate(Asp), phenylalanine (Phe), lysine (Lys), tyrosine (Tyr), second internal standard (IS2). CO_2 peaks of reference gas (Ref. Gas) are distributed throughout the chromatogram.

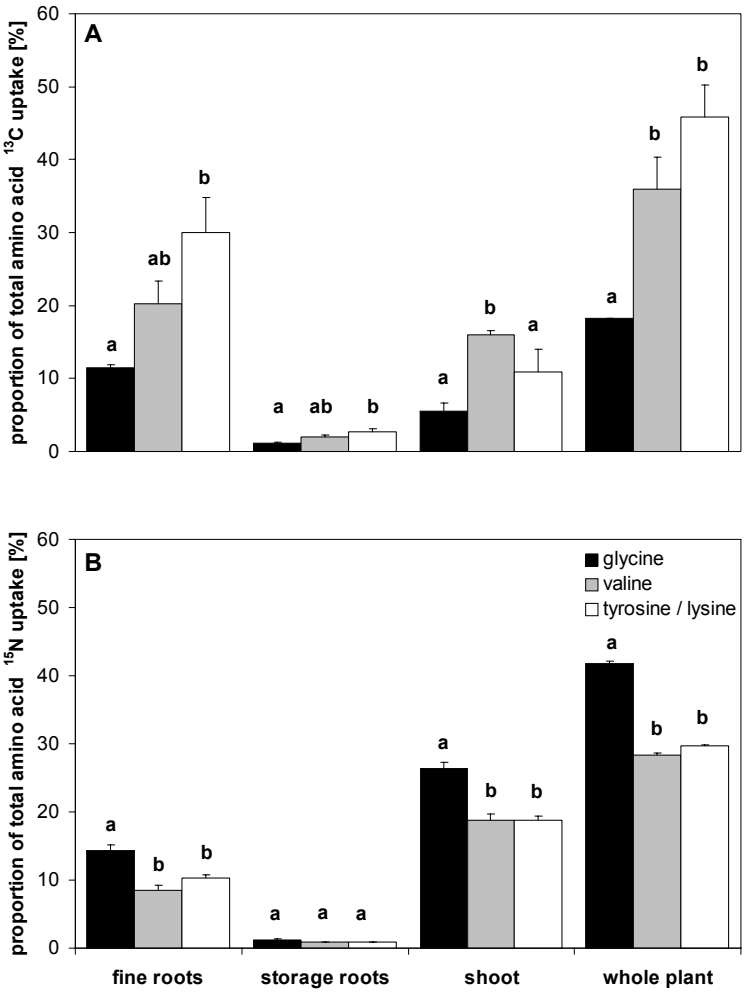

Figure 2.4: ^{13}C (A) and ^{15}N (B) enrichment of the target amino acids in each plant tissue based on the summed total ^{13}C and ^{15}N enrichment of all target amino acids for the whole plant, denoted as *proportion of intact amino acid ^{13}C / ^{15}N uptake*. Bars show mean values ± standard error; different lower case letters show significant differences between different amino acids for one plant tissue ($p<0.05$).

4 Discussion

4.1 Validity of compared data

In the calculation method introduced by Näsholm et al. (1998) the proportion of N taken up in the form of intact amino acids is calculated by comparing the ratio of ^{15}N : ^{13}C enrichment in the plant to the ^{15}N : ^{13}C ratio of the used tracer molecule. Therewith the authors take into account that ^{15}N can either be taken up as intact amino acid or as mineral N originated from microbial tracer decay in the soil. However, in this method ^{13}C uptake is considered more conservative i.e. if ^{13}C enrichment is detected in the plant it is attributed to the uptake of an intact amino acid C-skeleton. Therefore, we

deliberately compared the ^{13}C enrichments calculated from data of both measurements as any difference in this enrichment directly will lead to a difference in calculated uptake values for intact amino acids or rather amino acid N in case of the Näsholm calculation.

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

Compound-specific isotope measurements of amino acids in plants have been suggested as more accurate measure of amino acid uptake (Näsholm and Persson, 2001; Nordin et al., 2001; Persson and Näsholm, 2001). Still, even after corrections for the effects of derivatization C, amount dependency of isotope measurements and a calibration to international isotope standards, as it was done in our investigations, the at % ^{13}C values of the CSI measurements could still lead to a false estimation of the amino acid uptake. Selective plant measurements of ^{13}C enrichment in the type of amino acids used in the tracer mixture (target amino acids) avoids the problem of defining non amino acid bound ^{13}C uptake as uptake of intact amino acids as far as possible, but there still is a small chance of overestimating the amino acid uptake: If plants take up tracer-derived C fragments into the roots, those might be oxidized and fed into the tri carbonic acid cycle (TCA). By forming new amino acid skeletons this recycled ^{13}C theoretically can be incorporated into the target amino acids. However, this would also apply to all other newly synthesised amino acids and would lead to an enrichment of ^{13}C in non target amino acids. The highest enrichments should then be expected for those amino acids being synthesised directly from molecules of the TCA cycle and for those that are intensively consumed for N assimilation and N transport from root to shoot. In both cases these are glutamic acid, aspartic acid and their respective amides (Pate, 1980). However, we found no significant ^{13}C enrichment in aspartate or any other than the target amino acids (data not shown). Therefore, an overestimation of amino acid uptake is unlikely for CSI measurements.

It is also known that amino acids can be oxidised in the plant cells and fed back into the TCA cycle via an anaplerotic reaction (Marschner, 1995). If this is the case for a taken up tracer amino acid, the ^{13}C enrichment measured via CSI is likely to underestimate amino acid uptake in contrast to bulk measurements. Differences between both

measurements could thus be due to an underestimation of amino acid uptake in CSI measurements. Usually the re-feeding of amino acids into the TCA cycle happens after amino acids have been reallocated from mature plant tissues to sink organs like growing leaves or roots where they are transformed into molecules needed for the build up of new biomass (Bush, 1999). For root systems of mature plants, it is expected that unused amino acids are transported directly to the shoot (Bush, 1999; Lee et al., 2007). Our study was performed in August and sampled mature *P. lanceolata* plants with clearly visible young growing leaves. If oxidation of target amino acids took place after uptake we would expect a higher difference between bulk and CSI measurement in shoot compared to root tissues. Yet, our results show that differences between both methods are smaller for shoots than for fine roots, i.e. it is very unlikely that any significant oxidation of target amino acids appeared after uptake.

4.2 Overestimation of direct amino acid uptake by bulk measurements

We found a higher ^{13}C enrichment for all plant compartments with bulk compared to CSI measurements suggesting that this might be the result of one common process affecting all plant compartments. This leads to an overestimation of amino acid uptake as well as an overestimation of the proportion of intact N uptake using bulk measurements and the Näsholm equation as already reported by some authors (Nordin et al., 2001; Weigelt et al., 2003). The authors partly found relative direct uptake values for ^{15}N of more than 100% and Nordin et al. (2001) presented some thoughts on the possible causes of overestimation. The overestimation of amino acid ^{13}C uptake as found in our study did not lead to relative direct uptake rates of $^{15}N > 100$ %. However, the reasons for an overestimation of direct amino acid uptake as given by Nording et al. (2001) could potentially also apply to our investigation. The denoted authors investigated only shoot biomass and they ascribed their partly unrealistically high proportions of intact N uptake (> 100 %) to different compartmentation characteristics of amino acid C and N after uptake into the root. They suggested that most of the ^{15}N taken up as intact amino acid was transferred to the shoot after de- and transamination of the taken up tracer molecule while ^{13}C was incorporated into the root biomass (see Figure 2.1 C). Although our calculated intact N uptake using the Näsholm equation was within realistic ranges, we also found lower ^{13}C : ^{15}N enrichment ratios in shoot than in root material for bulk measurements which would support the ideas of Nordin et al. (2001). However, because of Nordin's results we deliberately did not only investigate shoot material but all plant compartments. As the ratio of ^{13}C to ^{15}N enrichment was not unrealistically

high in neither of these compartments we have to conclude that the supposed compartmentation might explain the high uptake rates by Nordin et al. (2001) but surely is not appropriate in our investigation.

Nordin et al. (2001) also suggested that the enrichment ratio between ^{13}C and ^{15}N in plant tissue might be influenced by microbial conversion processes of glycine before plant uptake (Figure 2.1 E). Microorganisms are able to transform two molecules of glycine into one molecule of serine plus ammonium and carbon dioxide via the glycine decarboxylase pathway (Oliver, 1994). If this process takes place in the plants' mycorrhiza, plants would take up serine with a C : N ratio of 3 : 1 instead of the originally applied glycine with a 2 : 1 ratio. Following Näsholm et al. (1998) the plant's enrichment ratio would then be compared to the ratio of the used tracer (2 : 1) which would mean that 150% of the taken up ^{15}N was taken up in form of amino acids. In addition the uptake of one serine instead of one glycine molecule would also lead to a higher absolute amount of ^{13}C uptake which could explain the higher ^{13}C enrichment of bulk measurements compared to CSI analysis in our investigation. However, we found no ^{13}C enrichment in serine for any of the plant compartments. This might either indicate that mycorrhizal amino acid uptake is of minor importance as suggested by Persson et al. (2001) or that the glycine decarboxylation in our investigations might not have been intense enough to influence the ^{13}C:^{15}N enrichment significantly. However, our data are not adequate to allow a decision on this question leaving alone the fact that no glycine dacarboxylation as suggested by Nordin et al. (2001) appeared in our investigations.

We believe that our investigations show that bulk measurements overestimate intact amino acid uptake as they imply uptake of ^{13}C as intact amino acid where in fact ^{13}C has been taken up only as amino acid fragment: As the major part of amino acids undergoes an incomplete breakdown via decarboxylation (Kuzyakov, 1997) followed by deamination and oxidation in the soil, the remaining products will be organic acids belonging to the low molecular weight (LMW) pool of soil. It has been suggested that plants do not only loose a vast number of this LMW compounds to the rhizosphere but might also compensate this loss via active uptake of these compounds (Farrar and Jones, 2000). Several studies have shown that plants are not only able to take up amino acids, but also carbohydrates and organic acids (Kuzyakov and Jones, 2006; Biernath et al., 2008) and that this uptake is not by chance or unspecific (Varanini et al., 1993). Any incomplete decay of ^{13}C labelled amino acids will therefore most likely lead to an up-

take of tracer derived ^{13}C enriched organic acids. However, bulk measurements can not differentiate between this ^{13}C uptake and the uptake of intact amino acid ^{13}C, thus leading to an overestimation of ^{13}C enrichment in bulk compared to CSI measurements.

The microbial metabolisation of amino acids in soil also produces CO_2 which accounts for a loss of up to 25 % of the applied tracer C within 24 h (Jones et al., 2005b). On its way to the soil surface this CO_2 can be dissolved in soil water by forming bicarbonate (Stumm and Morgan, 1996). Vuorinen et al. (1989) have shown that this bicarbonate can be taken up by plant roots and thereafter be incorporated into root biomass via the phosphoenolpyrovate carboxylase (PEP) pathway (dark fixation). Beside other abiotic and biotic factors, the activity of PEP has been shown to be mainly controlled by the amount of mineral N uptake (Sugiharto and Sugiyama, 1992b; Sugiharto and Sugiyama, 1992a; Manh et al., 1993; Diaz et al., 1996; Koga and Ikeda, 2000). In this context a number of authors showed that NO_3^- nutrition leads to higher contents in malat and other organic acid ions (Kirkby and Mengel, 1967; Van Beusichem et al., 1988; Luettge et al., 2000; Pasqualini et al., 2001). In detail Cramer et al. (1993) found that NH_4^+-dominated nutrition enhances uptake of bicarbonate followed by an equal distribution of the incorporated C to organic acid and amino acid synthesis with the highest enrichment in asparagine. On the other hand, predominant NO_3^- uptake led to a lower PEP activity and an increased incorporation of taken up C into organic acids. Our data provide no evidence for any amino acid enrichment other than the target ones. Thus, plants might have taken up mainly NO_3^-, bicarbonate fixation was low and ^{13}C enrichment was limited to organic acids. As Cramer et al. (1993) did not use labelled amino acids together with bicarbonate application, it remains unclear to which extent the dark fixation can explain the methodological differences in ^{13}C enrichment found in our investigations.

4.3 Additional information from CSI measurements

As bulk measurements can not differentiate between the uptake of single amino acids it demands the application of amino acid mixtures in which only one amino acid is labelled. For each additional amino acid to be investigated, a new treatment is needed with a different mixture of labelled amino acids. Therefore, it is not possible to directly compare the uptake characteristics of different amino acids in true replicates. CSI measurements can trace the uptake of single amino acids even if applied in a mixture. Our results show that less glycine was taken up compared to valine. This supports findings

of Sauheitl et al. (2009) but contradicts Lipson (1999a) who hypothesized that uptake rates of amino acids with lower C : N ratios are higher than for those revealing high C : N ratios.

CSI measurements also give information on the amount of tracer-derived ^{15}N fixed to the target amino acids. Näsholm et al. (1998) used bulk ^{15}N enrichment to determine the relative proportion of ^{15}N taken up in the form of amino acids to total ^{15}N uptake. Based on the fact that we measured ^{15}N enrichment in the target amino acids and showed that the ^{13}C enrichment in these amino acids reflects the amount of direct uptake, one should expect that the enrichment ratio of ^{13}C : ^{15}N in the target amino acids reflects the C : N ratio of the original tracer molecule. In contrast, our measurements show higher ^{15}N than ^{13}C enrichment leading to ^{13}C : ^{15}N ratios < 1 for glycine and < 3 for valine tyrosine and lysine in all plant tissues. In the bulk method this would imply that less than 100 % of ^{15}N fixed to the target amino acids had been taken up in an intact form. Basically there are two possible ways to explain this finding: Either the C skeletons of the tracer amino acids have been oxidized after uptake by the plant, or tracer-derived mineral ^{15}N has been added to newly synthesised target amino acids. In case of the former the low ratios of ^{13}C : ^{15}N enrichment in the target amino acids could only be explained if the carboxylic group was split off in an oxidative reaction catalysed by a peroxidase (Mazelis, 1962) leaving the amino group fixed to the remaining C skeleton. However, the amines formed in this reaction would no longer be identified as the original target amino acid which makes it necessary that the carboxylic group is added back to the same molecule in an anabolic reaction using a ^{12}C atom. In the case of glycine this would result in a ^{13}C : ^{15}N ratio of 1 : 1 instead of the original ratio of 2 : 1. Although this could explain the measured low ^{13}C : ^{15}N ratio just below 1 in storage roots, the whole taken up amount of tracer glycine would need to be run through the depicted reactions which is unlikely. Moreover, this process can not explain the enrichment ratios <1 of glycine in all other plant tissues or of the other target amino acids as these molecules have even higher C : N ratios than glycine.

While no ^{13}C enrichment was detected in any other than the target amino acids, which is a clear sign of the relatively low turnover of amino acid C in the plant, all of the 12 analysed amino acids showed significant ^{15}N enrichments in all plant tissues (data not shown). This can be explained by the fact that if any tracer-derived mineral ^{15}N is taken up by the plant it will partially be used for the formation of amino acids either in the shoot or in the root. In the case of NH_4^+ uptake, nitrogen will quickly be

assimilated in the form of amino acids due to its high plant toxicity (Marschner, 1995). As shoots only have a limited capacity for the disposal of protons (Raven, 1986) evolving from the assimilation reaction this is mainly done in the roots. As nitrate is not plant toxic it can either be stored in plant tissues after uptake or be transported to the shoot or be reduced to ammonium by the nitrate- and nitrite-reductase (Miflin and Lea, 1976a). In any case of ammonium emerging from plant uptake or transformation reactions, this ammonium will first be assimilated in the form of glutamate and glutamine via glutamate dehydrogenase or glutamate synthase and glutamine synthetase (Miflin and Lea, 1976b). Both molecules are central amino acids for the transport of nitrogen from root to shoot (Pate, 1980) and serve as N source for the synthesis of new amino acids. As the bulk of this synthesis takes place in the chloroplasts of photosynthetic active plant tissues (Bryan, 1990) it is very likely that a big part of the tracer-derived ^{15}N taken up is fixed to newly formed C skeletons in the shoot. This C is mainly derived from the glycolysis and therefore is largely composed of photosynthetically bound ambient CO_2 revealing natural abundance ^{13}C contents. Therefore, it can be expected that these newly formed amino acids dilute the ^{13}C enrichment found in the target amino acids leading to smaller ^{13}C : ^{15}N enrichment ratios in the target amino acids found in shoot tissues. We believe this to be the main mechanism explaining the generally low ^{13}C : ^{15}N ratio in target amino acids in all plant compartments as well as the significantly lower ^{13}C : ^{15}N ratio in shoot compared to root tissue. However, even the ratios found in the fine roots are too high to be caused by a pure uptake of intact amino acids which clearly shows that 24 h after label application newly formed amino acids have already been transported from the shoot to the root via the phloem (Bush, 1999).

5 Conclusions

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

1. Compound-specific isotope measurements of ^{13}C in the applied amino acids in plant tissues are an accurate indicator of direct plant amino acid uptake that is not affected by the uptake of tracer C-fragments.
2. In contrast, ^{13}C enrichment in bulk measurements was up to 8 fold higher than that of the CSI measurements leading to overestimations of direct amino acid uptake.

3. This overestimation is caused by the uptake of tracer derived ^{13}C-fragments. Possible molecules for this uptake are organic acids or bicarbonate ions, both originating directly or indirectly from tracer decay in soil. However, as already requested by Rasmussen et al. (2009) and Näsholm et al. (2009), further research is needed to show in detail which of these components account for most of the overestimation of amino acid uptake.
4. We were able to show that ^{15}N enrichment in the plants amino acids as derived from compound specific measurements can not be used to calculate the proportion of ^{15}N taken up in an intact form as done in the Näsholm equation. This is due to the high turnover of amino acid-bound ^{15}N and mineral tracer-derived ^{15}N in the plant.

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

Though our results show that the combination of bulk measurements with the Näsholm equation (Näsholm et al., 1998) tends to overestimate amino acid uptake in a soil of the temperate zone, this does not implement the inaccuracy of the method per se. It rather shows that Näsholm et al. (1998) specifically developed the method in soils with low annual mean soil temperature an low pH and therefore a low microbial activity. Future studies therefore should carefully check if this quick and simple method is really suitable for their kind of environment.

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Study 3: Plant uptake of dual-labelled organic N biased by inorganic C uptake: results of a triple labelling study

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Abstract

Direct plant uptake of organic nitrogen (N) is often studied using the dual labelling approach ($^{15}N+^{13}C$ or $^{15}N+^{14}C$). However, the method might be hampered by uptake of labelled inorganic carbon (C) produced by mineralization of labelled organic compounds. Here we report the results from a triple labelling experiment ($^{15}N+^{13}C+^{14}C$) investigating whether root uptake of labelled inorganic C can bias the results obtained in studies of organic N uptake using dual labelled amino acids (^{15}N, ^{13}C). In a rhizosphere tube experiment we investigated ^{13}C and ^{14}C uptake by maize either supplied with labelled glycine or CO_3^{2-}, but found no differences in uptake rates between these C-sources. The uptake of inorganic C to the shoot tissue was higher for maize grown in full light compared to shading, which indicates a passive uptake of inorganic C with water. We conclude that uptake of inorganic C produced by mineralization of amino acids can significantly bias the interpretations of organic N uptake studies using dual labelling.

Keywords: organic N uptake, amino acid, inorganic C, dual labelling, ^{14}C, ^{13}C, ^{15}N

1 Introduction

For many years plant N acquisition have been assumed to mainly occur in the inorganic N forms, NO_3^- and NH_4^+. The finding of Näsholm et al. (1998) that plants take up N in organic form renewed the discussion of sources for plant N acquisition. Various methods have been used to document plant uptake of organic N such as amino acid depletion from liquid cultures (El-Naggar et al., 2009), bulk measurement of dual labelled amino acid uptake (Fokin et al., 1993), and compound specific isotope analysis of intact dual labelled amino acids in shoots and roots (Näsholm et al., 2001; Sauheitl et al., 2009a). Bulk measurement of uptake of dual labelling with ^{13}C (or ^{14}C) and ^{15}N has been used for the last decade in a great number of studies (e.g. Streeter et al., 2000; Weigelt et al., 2005; Sauheitl et al., 2009b). The prerequisite is that the total amount of labelled C recovered in the plant corresponds to the amount of labelled amino acid taken up by the plant, and not in form of C mineralized to CO_2. However, root uptake of inorganic C has been studied since the 1950 (Graf and Aronoff, 1955) and the incorporation of inorganic C into plant tissue via phosphoenolpyruvate (PEP) carboxylase is also well described (Vuorinen et al., 1992; Britto and Kronzucker, 2005). We recently addressed the possibility that the uptake of labelled inorganic C might bias the results from organic N uptake studies when using bulk measurement of dual labelling (Rasmussen and Kuzyakov, 2009). In the present study we aimed to test the significance of this inorganic C uptake for studies on organic N uptake using bulk measurement of C and N dual labelling. That is, if uptake of added inorganic tracer C is in a similar order as added organic tracer C, it would be impossible to distinguish which process caused the tracer C to enter the plant. Thus, the prerequisite assumption that the total amount of labelled C recovered in the plant tissue originates from uptake of the added organic compound is not valid.

2 Material and Methods

We performed a triple labelling study with maize seedlings grown in rhizosphere tubes (Jones et al., 2005; Biernath et al., 2008) to study the uptake of ^{14}C, ^{13}C, and ^{15}N applied to the soil in inorganic and organic form. Our experiment resembled that of Biernath et al. (2008), using the same soil and experimental setup, except that in the present study we used glycine and excluded ventilation of the rhizosphere tubes in order to maintain the partial pressure of CO_2 in the soil. The soil was a loamy

haplic Luvisol taken from the A_p horizon at the University of Hohenheim Agricultural Research Station at Fildern, Stuttgart, Germany. The soil had a pH (H_2O) of 6.9, pH ($CaCl_2$) of 6.5, C_{tot} and N_{tot} contents of 1.5% and 0.14%, respectively (additional information are given in Biernath et al. (2008)). The roots of maize seedlings grown in rhizosphere tubes were sealed off at the soil surface with silicone rubber paste before amended with a labelling solution of either:

(A) U-[^{13}C]-[^{15}N]-glycine + U-[^{14}C]-glycine, or

(B) U-[^{13}C]-[^{15}N]-glycine + $Na_2{}^{14}CO_3$, or

(C) $K^{15}NO_3$ + $Na_2{}^{13}CO_3$ + $Na_2{}^{14}CO_3$

with N concentration of 0.13-0.15 µg N g^{-1} soil and C concentrations for solution (A) and (B) of 0.22-0.23 µg C g $soil^{-1}$ and 0.11 µg C g $soil^{-1}$ for solution (C) (Table 3.1) in order to resemble a 50% release of tracer C from mineralization of the organic compound. Nitrate ($K^{15}NO_3$) was used as the inorganic tracer N source in solution (C) as ammonium has been shown to enhance the dark incorporation of inorganic C in maize roots compared to nitrate (Cramer et al., 1993). Thus nitrate was the conservative choice of tracer N in relation to inorganic C uptake. After addition of the labelling solution plant were grown in a growth chamber under either full light (295 µmol m^{-2} s^{-1}) or shading (55 µmol m^{-2} s^{-1}) in order to study the impact of reduced water uptake on the uptake of tracer C. Maize plants were destructively sampled 24 hours after labelling and divided into shoots and roots. We chose to sample the plants after 24 hours in order to reduce a possible volatilization of CO_2 added as Na_2CO_3 or produced from respiration and mineralization of glycine. Untreated control plants were grown between labelled plants in order to correct for incorporation of ^{14}C and ^{13}C-labelled CO_2. Root and shoot samples were grinded to fine powder before analysis for ^{14}C-activity by combustion in a Packard model 307 sample oxidizer (Packard Instrument Company, Meriden, CT, USA) and ^{13}C and ^{15}N enrichment by an elemental analyzer (Carlo Elba, NC 2500) coupled with IRMS (Delta^{plus}, Thermo, Bremen, Germany). All data were analyzed using the GLM procedure of SAS (SAS Institute, 1999) in a two-way analysis of variance with tracer solution and lighting conditions as fixed effects.

Table 3.1: Characteristics of labelling solutions. A: U-[^{13}C]-[^{15}N]-glycine + U-[^{14}C]-glycine, B: U-[^{13}C]-[^{15}N]-glycine + $Na_2{}^{14}CO_3$, C: $K^{15}NO_3$ + $Na_2{}^{13}CO_3$ + $Na_2{}^{14}CO_3$.

	A	B	C
Added N concentration in soil (µg N g soil^{-1})	0.13	0.13	0.15
Added C concentration in soil (µg C g soil^{-1})	0.22	0.23	0.11
^{15}N abundance (%)	98.00	98.00	10.40
^{13}C abundance (%)	98.00	98.00	97.90
Total ^{14}C activity added per rhizosphere tube (kBq)	126.00	180.00	181.00

3 Results and Discussion

Tracer C (^{13}C and ^{14}C) from all three labelling solutions was found in maize shoot and root tissues (Figure 3.1, Table 3.2). We found no significant differences in the total uptake (% of added) of tracer C in shoot or root tissue between glycine and carbonate application (Figure 3.1), whatever the light treatment. Total uptake of tracer N in root tissue differed significantly ($p = 0.0003$) between the three labelling solutions in the order B > A > C, irrespective of light treatment. Simultaneous uptake of tracer C and tracer N from glycine (Figure 3.1, Table 3.2) is believed to show the uptake of intact organic compounds (Näsholm et al., 1998) and the conclusions of many studies are based on this assumption. Näsholm et al. (1998) investigated the uptake of glycine in a forest soil with a pH of 3.1. At such a low pH, the concentration of dissolved bicarbonate and carbonate is very small and should therefore not influence the calculated intact amino acid uptake. However, in a number of later studies, soil pH has been above 5 (e.g. Näsholm et al., 2000; Weigelt et al., 2005; Kielland et al., 2006) and in the present study it was 6.9 (in H_2O). At this pH inorganic C can be dissolved in significant amounts (see supporting online material in Rasmussen & Kuzyakov, (2009) in soil water and - as shown here - taken up by the plants.

The half-lives of amino acids in soil are within hours (Jones et al., 2005). This implies that for example glycine will be rapidly mineralized to its inorganic components, as shown by the presence of labelled CO_2 in controlled experiments (Biernath et al., 2008; Jones et al., 2005). In the Biernath et al. (2008) study, less than 1 % of the ^{14}C added in the form of alanine was taken up by the plants, whereas more than 50 % was recovered as $^{14}CO_2$ in air leaving the root system after 24 hours. In the present study, 5-10 % of the ^{14}C added was recovered from the shoot and root tissue after 24 hours of labelling. We explain the difference in the recovery of ^{14}C in the plant tissue between the two studies as being due to a reduction in the partial pressure of CO_2 in soil due to

ventilation of the rhizosphere tubes in the Biernath et al. (2008) study, although differences in plant preference for the used amino acids may also influence the interpretation (Jones, 1999; Sauheitl et al., 2009b). The closed soil system used in the present study most likely invoked soil air to have a higher partial pressure of CO_2 from added CO_3^{2-} or mineralized glycine, than would be found in an open soil system, causing a likely overestimation of the inorganic tracer C uptake. However, the high uptake rate of tracer C clearly shows that in this study it is impossible to distinguish if tracer C entered the plant in organic or inorganic form; this to greater or lesser extent also applies to more open systems depending on the soil air ventilation. Thus, the prerequisite assumption of dual labelling bulk measurement studies of organic N uptake is not valid.

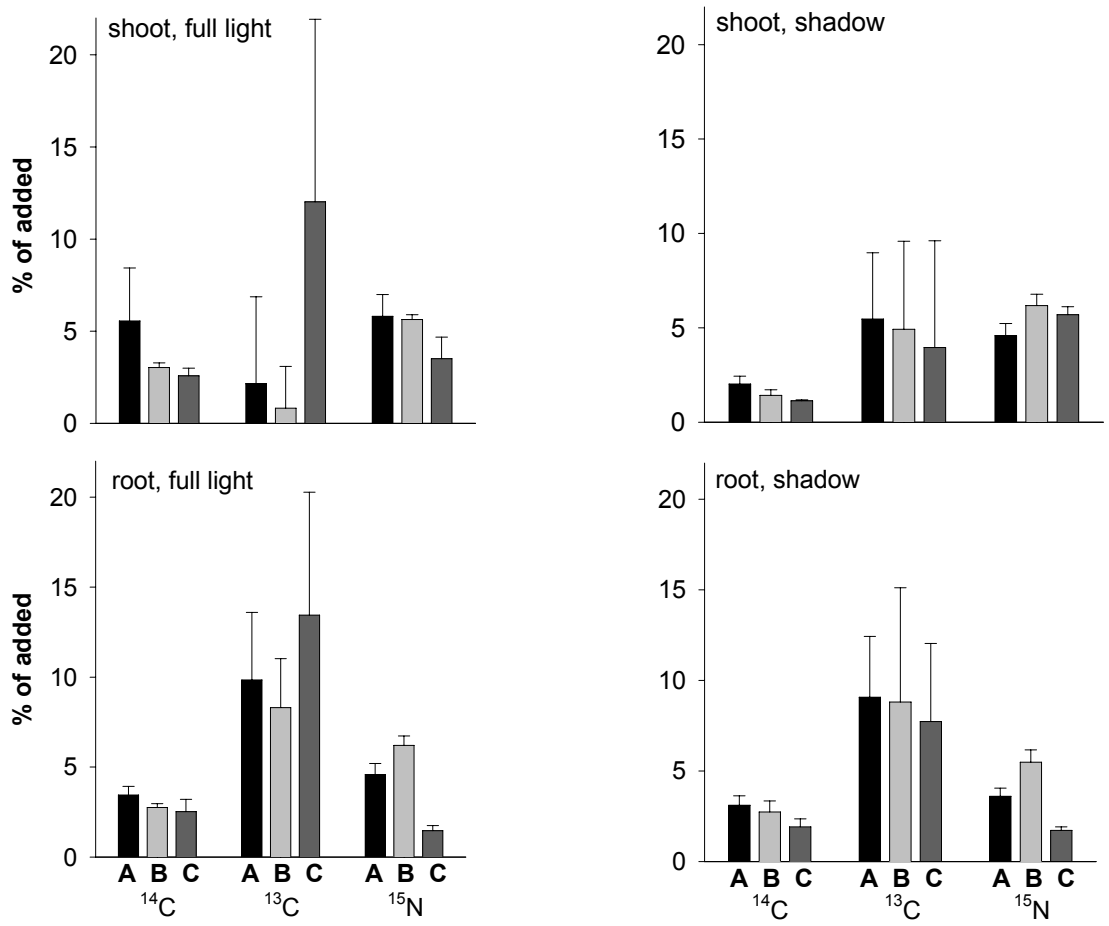

Figure 3.1: Uptake of ^{14}C, ^{13}C, and ^{15}N in percent of added tracer in maize shoot and root tissue after 24 hours of labelling in either full light or shading. Treatment A: all tracers in the form of glycine; B: ^{13}C and ^{15}N in glycine form and ^{14}C in carbonate form; C: all tracers in inorganic form. Error bars show the standard error based on four replicates.

In the present study we found a significantly ($p = 0.039$) higher total uptake of ^{14}C in shoot tissue under full light than under shading independent of the labelling solution. The total uptake of ^{13}C or ^{15}N in shoot and root tissues was not significantly dif-

ferent between full light and shading, although the variability for ^{13}C was so high that a possible biological effect could have been masked. The uptake of ^{14}C from the carbonate tracer is in accordance with the study of Vuorinen et al. (1989) who found ^{14}C incorporation in willow from $NaHCO_3$ to be twice as high after 24 hours in light than in darkness. Based on the ^{14}C uptake we suggest that the uptake of inorganic C in the shoot supports the conclusion of Vuorinen et al. (1989) that a part of the uptake is driven by transpiration and the increased water flow from root to shoot under full light.

Table 3.2: Dry matter yields, ^{14}C-activity, ^{13}C- and ^{15}N-enrichment, and uptake rates under full light or shading for shoot and root tissues of maize plants labelled with solution (A), (B) and (C).

			Yield g DM tube^{-1}	**^{14}C-activity** kBq g DM^{-1}	**^{13}C-excess** nmol g DM^{-1}	**^{15}N-excess** nmol g DM^{-1}
Shoot tissue	full light	A	0.11±0.01 a	68±31 a	50±88 a	108±16 ab
		B	0.12±0.01 a	51±3 ab	12±35 a	98±14 b
		C	0.12±0.02 a	47±6 ab	76±82 a	37±16 c
	shading	A	0.10±0.01 a	27±4 b	109±71 a	100±17 b
		B	0.09±0.02 a	30±2 ab	62±92 a	154±24 a
		C	0.09±0.01 a	25±3 b	54±60 a	67±4 bc
Root tissue	full light	A	0.06±0.01 a	79±2 a	338±108 a	164±9 ab
		B	0.07±0.01 a	76±5 a	240±65 a	194±31 a
		C	0.08±0.02 a	58±3 b	135±59 a	21±5 c
	shading	A	0.06±0.01 a	69±4 ab	290±79 a	130±13 b
		B	0.08±0.01 a	65±3 ab	180±157 a	153±9 ab
		C	0.07±0.01 a	52±8 b	111±69 a	27±3 c

The results of the present triple labelling study demonstrated that uptake of tracer-C in inorganic form can bias the results in studies using bulk measurement of dual-labelling to show organic N uptake. Our findings imply that the conclusions from many such studies of organic N uptake have to be reconsidered, as control treatments using inorganic C tracers have rarely been included. We anticipate that in the future, studies of organic N contribution to plant N nutrition will need to make use of compound specific isotope methods able to track the flow of intact organic N compounds and their metabolites from soil into the plant tissue.

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Study 4: Amino acid fingerprint of soils reflects plant diversity changes in a grassland ecosystem

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Abstract

A positive plant diversity to plant aboveground productivity relation has been shown to alter carbon and nitrogen fluxes in soils. Thus, most investigations focussed on the C- and N-input via litter fall, widely neglecting the importance of root exudation. As microbes, which are known to be important drivers of matter fluxes in soil, feed on these root exudates, increased knowledge on the availability of these compounds in soil might help to understand plant biodiversity effects on soil.

We therefore investigated the effect of plant diversity on size and composition of the free soil amino acid pool in a grassland experiment, as amino acids are an important C- as well as N-source for microbes.

Despite a positive diversity effect on plant productivity, we only found an insignificant increase of the size of the free amino acid pool in soil. This was most likely caused by an increase of the microbial population and thus an increased amino acid mineralization. At the same time the composition of the amino acid pool changed significantly between plant diversity levels. This most likely reflects differences in plant input as well as differences in microbial mineralization and enabled us to separate diversity levels by means of discriminant analysis.

Keywords: Amino acids, fingerprint, diversity, plants, grassland

1 Introduction

One central and very frequent finding in plant biodiversity research is the positive effect of plant diversity on plant productivity. This effect has been described and discussed in a number of studies (Tilman et al., 1996; Hector et al., 1999; Loreau et al., 2001; Hooper et al., 2005; Spehn et al., 2005; van Ruijven and Berendse, 2005; Marquard et al., 2009) and research during the last years began to focus on the mechanisms behind this correlation as well as on the effects of the higher plant productivity on system variables (Tilman et al., 1996; Tilman et al., 1997; Loreau, 1998a; Chapin et al., 2000; and citations therein). Higher plant productivity implicates a higher C-input into soil (Gleixner et al., 2005; Steinbeiss et al., 2008) and might thus alter fluxes and pool sizes (Hooper et al., 2005) in the carbon and the associated nitrogen cycle. In contrast, a mechanistic model by Loreau (Loreau, 1998a) suggested that soil processes are not necessarily influenced by increasing plant productivity. This shows that depending on the system and the variables investigated, the impact of plant diversity and plant productivity can differ in their significance and that more data from field experiments are needed to get a full picture of the possible effects of plant diversity. Most of the C-input into a system is derived from litter fall and root exudation (Catovsky et al., 2002) where the latter one is mainly comprised of carbohydrates and amino acids (Kraffczyk et al., 1984; Farrar et al., 2003). The bulk of the microbial community feeds on these low molecular weight substances (Haller and Stolp, 1985) and therefore its size and metabolic activity is highly related to the rate and composition of root exudation. In this, amino acids as the dominant organic N form in root exudates (Merbach et al., 1999; Hütsch et al., 2002) and soil (Kelley and Stevenson, 1996; Fischer et al., 2007) play a key role in the microbial nutrition as it has been shown that microbes located in the rhizosphere are rather N- than C-limited (Kuikman et al., 1990; Liljeroth et al., 1990; Breland and Bakken, 1991). Therefore, plants might be able to affect size and composition of the microbial soil community as found by Hartman et al. (2009) and thus influence one of the most important organisms with respect to C- and N-fluxes in soil (van der Heijden et al., 2008). Despite this importance of amino acid availability in the rhizosphere, little is know about effects of plant diversity on free soil amino acid concentrations and models on the interaction between producers (plants) and decomposers (microbes) lack in the use of this information (Loreau, 1998b; Loreau et al., 2001).

We therefore investigated the effect of increasing plant diversity accompanied by increasing plant productivity on amino acid concentrations in soils of a grassland experiment and hypothesised that:

1) The size of the free amino acid pool in soil will increase with increasing plant diversity as it has been shown that root exudation is mainly controlled by the size of photosynthetically active plant tissues (Kuzyakov and Cheng, 2001).
2) The contribution of single amino acids to the total amino acid pool in soil will differ between plots of differing plant species richness as the composition of root exudates can differ between plant species (Hertenberger and Wanek, 2004). This will form an amino acid fingerprint in soil that is characteristic for each investigated species richness level.

2 Methods

2.1 Soil sampling

Soil was taken from plots of the Jena Experiment (Roscher et al., 2004). The field site is located on a flood-plain of the Saale river near Jena, Germany (11°34'60" East; 50°55'60" North; altitude 130 m a.s.l.) and consists of grassland plots differing in plant diversity. In this study we used 28 plots of 3.5 x 3.5 m. Plant diversity changed with the number of species from 1 to 2, 4, 8 and 16 and the number of plant functional groups from 1 to 2 and 3. The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was highly fertilized during the last 40 years of agricultural utilisation until 2002 when the Jena Experiment started. Soil was sampled in August 2007 with a metal corer (5 cm diameter) to a depth of 30 cm representing the main root horizon. On each plot 4 soil samples were taken, merged and sieved to < 2 mm before they were stored at -40 °C until further analysis.

2.2 Amino acid extraction, purification and derivatization

The extraction procedure combines the procedures proposed by Houba et al. (1986) and Mengel et al. (1999) with small changes in extraction time and the use of internal standards. Soil was dried using a freeze dryer (Christ Beta 1-8, Germany) and approximately 15 g of the dry soil were filled into glass flasks. For extraction of free and adsorbed amino acids 100 mL 0.01 M $CaCl_2$ solution that was heated to 80 °C beforehand were added to the soil together with 300 nmol of L-Norvaline (Phenomenex, Torrance, CA) acting as first internal standard. The suspension then was shaken for

20 min. at 80 °C in a water bath (Julabo SW22) at an intensity of 95 rpm. The soil was separated from the solution by centrifugation for 15 min. at 2000 rpm and the supernatant was transferred to 250 mL conical flasks. The extraction solution then was concentrated to app. 2 mL using a rotary evaporator (T < 45°C and p = 40 mbar). The resulting solution was transferred to a 5 mL glass flask and the wall of the conical flasks was rinsed again with 2 x 1 mL of 0.05 M HCl which was also transferred to the glass flasks.

An aliquot of 200 µl of this concentrate was then processed with the amino acid analysis kit EZ:faast® (Phenomenex, Torrance, CA, USA). In this procedure amino acids are purified using solid phase extraction of amino acids to separate them from metal cations and anionic organics of the original solutions. In the last step of the procedure the purified amino acids were transformed to formic acid esters that can be measured on the GC and were then transferred to gas chromatograph vials. The instructions for the procedure were provided by the manufacturer and are explained in detail elsewhere (Kugler et al., 2006). Previous to the esterification, 27 nmol of trans-4-(aminomethyl) cyclohexane carboxylic acid (Sigma Aldrich) were spiked to the sample solution acting as second internal standard. Together with the samples, various amounts of an amino acid standard (1, 2, 4 and 10 nmol of each amino acid) containing 19 amino acids (Phenomenex) were processed and derivatized along with the samples.

2.3 Measurements

Chromatographic separation and quantification of the single amino acids was done using a HP 6890 Series gas chromatograph (GC) coupled to a flame ionization detector (FID). A Zebron ZB-AAA GC-column (10 m x 250 µm x 10 µm, Phenomenex) was used for peak separation and temperature and flow conditions of the column were optimized to allow for optimal peak separation. 2 µL of each sample and standard were injected into the injector of the GC, respectively. Injector temperature was set to 250 °C and column flow was adjusted to 1.5 mL min^{-1}. Starting temperature of the GC oven was 110 °C instantly raised to 320 °C at a rate of 25 °C min^{-1} and detector temperature was set to 320 °C. Peak separation of all amino acids was complete and 13 free amino acids could be frequently detected in all samples (Table 4.1).

Table 4.1: List of investigated amino acids, including their complete and short names and their order of appearance in the chromatogram.

Amino acid	Short name	Time (min.)
Amino acids in the extracts		
Alanine	Ala	1.54
Glycine	Gly	1.65
Valine	Val	1.84
Norvaline[a]	Nor	1.96
Leucine	Leu	2.04
allo-Isoleucine	alle	2.07
Isoleuciene	Ile	2.10
Proline	Pro	2.42
Aspartic acid	Asp	3.03
Glutamic acid	Glu	3.37
Phenylalanine	Phe	3.41
trans-4-(aminomethyl) cyclohexane carboxylic acid[b]	ACC	3.76
Ornitine	Orn	4.61
Lysine	Lys	4.79
Histidine	His	5.06
Not detected amino acids		
Serine	Ser	n.d.
Metionine	Met	n.d.
Glutamine	Gln	n.d.
Asparagine	Asn	n.d.

[a]: First internal standard, not present in nature.

[b]: Second internal standard, not present in nature.

2.4 Calculations and Statistics

Amino acid content is indicated as amount of amino acids in nmol * g^{-1} dry soil, taking the specific recovery of each sample into account. Relative amino acid content (*% amino acid content*) was calculated as the content of a specific amino acid based on the total amino acid content of the sample (1).

$$\% \text{ amino acid content} = \frac{\text{amino acid content}_{i,\,x}}{\text{total amino acid content}_{x}} \qquad (1)$$

Where *amino acid content*$_{i,\,x}$ is the content of an amino acid *i* in a sample *x* and *total amino acid content*$_x$ represents the total content of all amino acids in a sample *x*.

All following statistical analysis were done with the SPSS package 10.0.1. (SPSS GmbH, Munich, Germany). Total and relative amino acid values of each amino acid were tested to be normally distributed and variances between groups to be equal using the Shapiro-Wilk and the Levene test, respectively. Significant changes of the *amino acid content* and the *% amino acid content* between diversity levels for single amino acids and significant changes of both variables between different amino acids for

single diversity levels were tested using the analysis of variances (ANOVA) procedure followed by a Scheffé post-hoc test. If variances were not equal between groups, a Kruskal-Wallis test was performed followed by a Games-Howell post-hoc test. A discriminant analysis was conducted using either *amino acid content* or *% amino acid content* as independent variable and species richness as grouping variable. Equal covariance of the populations in the discriminant analysis was tested using the Box test and the significance and explanatory power of the discriminant function was tested using Wilks' Lambda. Significant differences between the standardized canonical discriminant function coefficients between diversity levels were tested with an ANOVA followed by a Scheffé post-hoc test.

3 Results and Discussion

The size of the soil amino acid pool (*total amino acid content*) showed a rising trend from low diversity to high diversity (Figure 4.1), though this trend was not significant (ANOVA, $p < 0.05$). Changes of the amino acid content for single amino acids between different diversity levels where small and lysine was the only amino acid to change significantly (ANOVA, $p < 0.05$) between diversity levels (Figure 4.2A). These small changes are in accordance with trends reported in literature (Bremner, 1966; Stevenson, 1982). These authors suggested that neither the total amino acid content nor the content of single amino acids is affected by soil type or ecosystem changes like land use practice and therefore might not be a feasible way to trace environmental changes in soil. Our results seem to confirm this point of view at first glance. This contradicts our first hypothesis in which we assumed that a higher aboveground plant biomass will result in higher exudation rates due to higher photosynthesis (Kuzyakov and Cheng, 2001) and therefore in a higher concentration of free amino acids in soil. In this context it has be noted that soil amino acid concentrations are not only affected by exudation rates but also by the size and activity of the microbial population feeding on this substrate. Investigations of the microbial community in the same experiment indeed showed that there was an increase in size (Sauheitl et al., 2009a; in preparation) and turnover (Sauheitl et al., 2009b; in preparation) of the microbial community with increasing plant diversity. This implicates an increasing N demand of soil microbes and might thus result in a damped increase of total amino acid contents, lacking in significance, as it was found in our investigations.

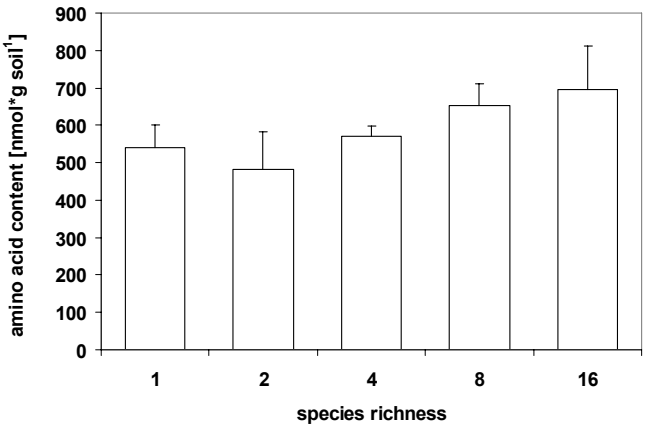

Figure 4.1: Total content of all analyzed amino acids in nmol*g^{-1} soil for different numbers of plants (1 – 16) indicated as species richness (SR). Bars show mean values ± standard error.

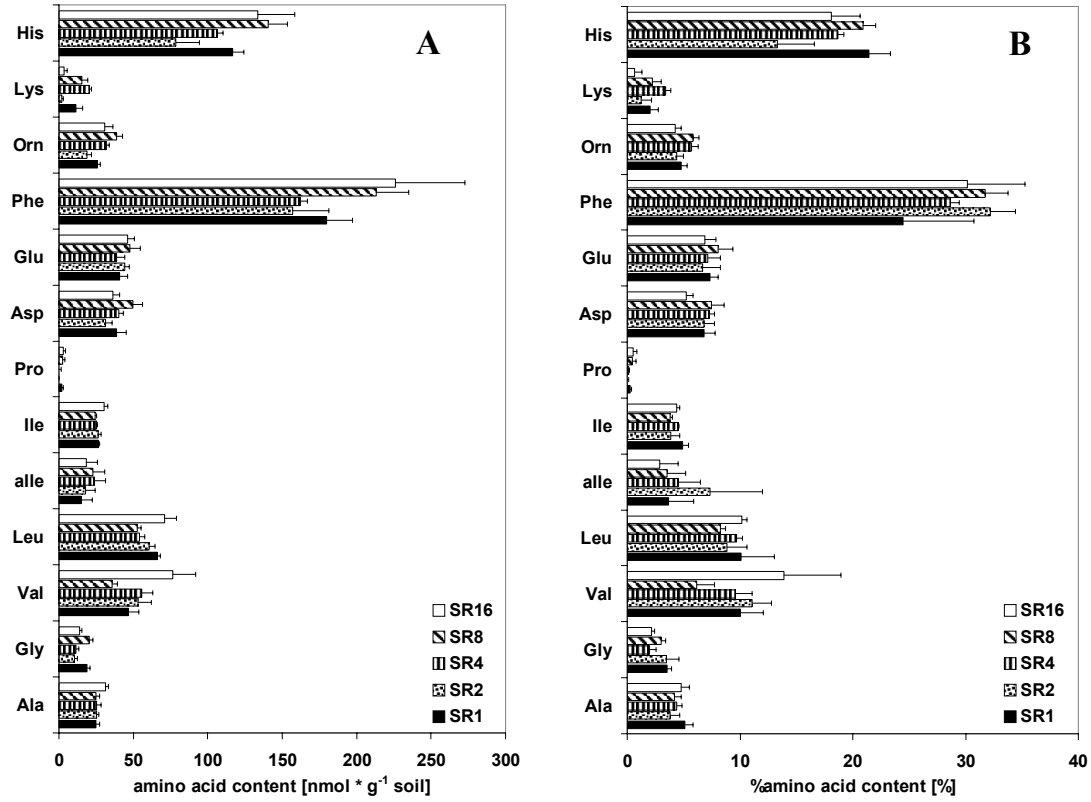

Figure 4.2: Absolute content of single amino acids in nmol*g^{-1} soil (A) and content of single amino acids based on the total content of all amino acids (*% amino acid content*) (B) for different numbers of plants (1 – 16) indicated as species richness (SR). Bars show mean values ± standard error. Amino acid contents are depicted as open bars for 1 species mixtures, diagonal hatched bars for 2 species, vertical hatched bars for 4 species, dotted bars for 8 species and solid bars for 16 species mixtures. Abbreviations for amino acids are given in Table 4.1.

However, differences between the concentration of amino acids within single diversity levels were significant for all diversities (ANOVA, $p < 0.05$). While phenylalanine and histidine were the dominant amino acids and lysine and proline were the minor amino acids the for all plant species richness levels, the order of all other amino acids changed between diversity levels (Table 4.2 A). This was also true when the effect of the *total amino acid content* was eliminated (Figure 4.2B, Table 4.2 B). However, the amino acid fingerprint resulting from *% amino acid content* might be a more suitable tool for direct comparisons between different biodiversity levels as it eliminates the effect of very high or low amino acid concentration, leaving the hierarchical structure in the amino acid pool as the dominant information. This procedure was first used by Beavis et al. (1996; 1999) to distinguish between soils from different land use systems and different manuring practices.

Table 4.2: ANOVA table of differences between *amino acid content* (A) and *% amino acid content* (B) for single species richness level (SR), respectively. Different letters show significant differences ($p < 0.05$) between amino acids within a species richness level and numbers indicate size ranks of amino acids within a species richness level.

	SR	Ala	Gly	Val	Leu	alle	Ile	Pro	Asp	Glu	Phe	Orn	Lys	His
A)	1	cd	cd	c	c	cd	cd	d	cd	cd	a	d	d	b
		9	10	3	4	11	7	13	6	5	1	8	12	2
	2	bc	bc	bc	bc	bc	bc	c	bc	bc	a	c	c	ab
		8	11	4	3	10	7	13	6	5	1	9	12	2
	4	cde	de	c	c	cde	cde	e	cd	cd	a	e	cde	b
		8	12	3	4	10	9	13	5	6	1	7	11	2
	8	b	b	b	b	b	b	b	b	bc	a	b	b	a
		8	11	7	3	10	9	13	4	5	1	6	12	2
	16	b	b	ab	ab	b	b	b	b	bc	a	b	b	ab
		8	11	3	4	10	9	13	6	5	1	7	12	2
B)	1	c	c	ab	ab	c	c	c	bc	bc	a	c	c	ab
		9	10	3	4	11	7	13	6	5	1	8	12	2
	2	bc	c	bc	bc	bc	bc	c	bc	bc	a	c	c	b
		8	11	4	3	10	7	13	6	5	1	9	12	2
	4	cde	de	c	c	cde	cde	e	cd	cd	a	e	de	b
		8	12	3	4	10	9	13	5	6	1	7	11	2
	8	cde	cde	cde	c	cde	cde	de	cd	cd	a	e	cde	b
		8	11	7	3	10	9	13	4	5	1	6	12	2
	16	bc	c	bc	bc	bc	bc	c	bc	bc	a	c	c	ab
		8	11	3	4	10	9	13	6	5	1	7	12	2

Though changes in the relative amount of amino acids between the sites used in the studies of Beavis et al. were not always significant using ANOVA, the authors managed to separate the different sites using a discriminance analysis. The soils investigated by Beavis et al. had already been shown to change in organic N content

(Jenkinson and Rayner, 1977) and given the established correlation between amino acid and organic N concentration (Beavis and Mott, 1996) a change in amino acid concentration had to be expected. However, the authors intended to develop a method of tracing land use change independent of altering total amino acid concentration. Therefore they removed the effect of changing total amino acid concentrations by using relative concentrations of single amino acids based on the total amino acid content. As our second hypothesis should be investigated independent of the results for the first hypothesis, concerning the total amount of amino acids, we also followed this procedure. The four discrimant functions adapted to the data in the analysis explained 100 % of the variations in the amino acid contents (Table 4.3). The low value of Wilks' Lambda (Table 4.4) suggests that the means of the four functions are different between diversity levels, which indeed was highly significant ($p < 0.001$). The scatter plot of functions 1 and 2, which explained 93 % of the observed observations, shows that there is a small scatter of single plot values around the respective group centroid (Figure 4.3). Using all four functions, the discriminant analysis reports a complete separation of all species richness levels from one another without any false classifications. This was confirmed by the results of an ANOVA on differences between the groups standardized canonical discriminant function coefficients (data not shown). In detail the ANOVA revealed, that the first function, accounting for 74 % of the total variance, separates groups SR2 and SR8 from each other and from the remaining groups SR1, SR4 and SR16. The remaining group separations are achieved using equations 2 and 4 while equation 3 accounts for variations within groups as its function coefficients do not differ between groups.

Table 4.3: Eigenvalue, explained percentage of variance and canonical correlation of the four functions using *% amino acid content* (B) as independent variable.

Function	Eigenvalue	% of Variance	Cumulative %	Canonical Correlation
1	13.156[a]	74.1	74.1	0.964
2	3.345[a]	18.8	93.0	0.877
3	0.702[a]	4.0	96.9	0.642
4	0.542[a]	3.1	100.0	0.593

[a]: First four canonical discriminant functions were used in the analysis.

Table 4.4: Wilks' Lambda, Chi-square, degrees of freedom (df) and significance value of combinations of the discriminant functions as determined in the discriminant anaylsis using *% amino acid content* as independent variable.

Test of Function(s)	Wilks' Lambda	Chi-square	df	Sig.
1 through 4	0.006	83.886	48	0.001
2 through 4	0.088	40.160	33	0.183
3 through 4	0.381	15.922	20	0.721
4	0.648	7.148	9	0.622

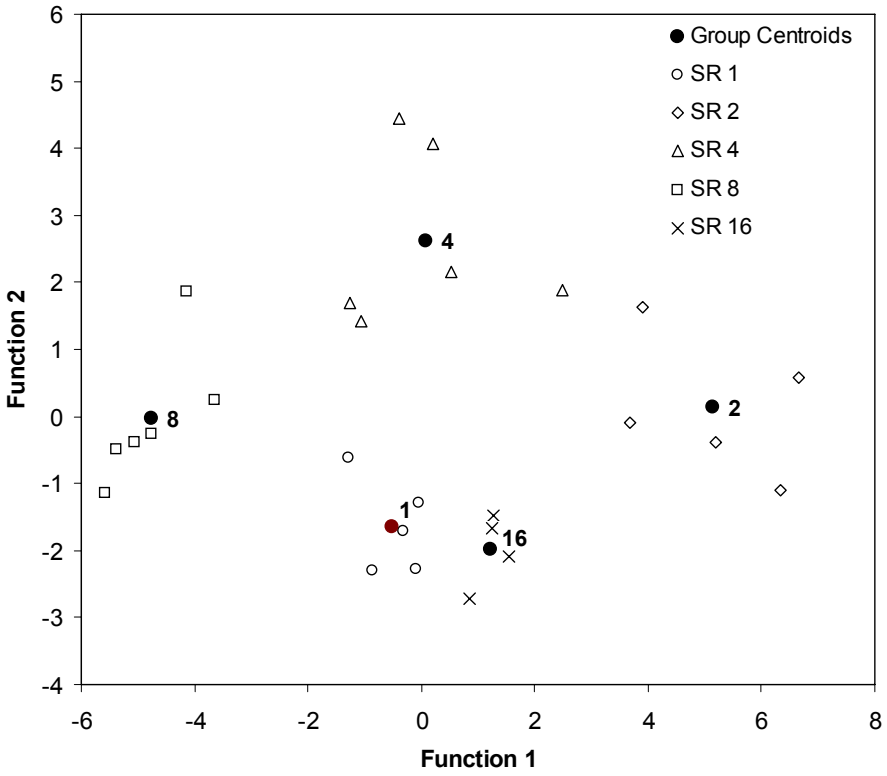

Figure 4.3: Scatter plot of the relative amino acid (*% amino acid content*) composition of soils with different plant diversity as indicated by the first two discriminant functions. Solid symbols show group centroids and open symbols show single values of each species richness level.

Beavis et al. (1996; 1999) tested the applicability of the amino acid fingerprint method under conditions where treatment effects, e.g. manuring or land use, are large in relation to variations of other soil variables. The equations calculated in their discriminance analysis were therefore clearly referred to one of the treatment effects, i.e. accounting for time span of treatment or type of land use. The separative effect of each discriminant function therefore could be ascribed to one defined treatment. In contrast to that our experiment only manipulated one factor, namely plant diversity, by means of altered numbers of plant species. Though it is known that plant diversity affects the ecosystem via a number of top down and bottom up effects that influence

soil conditions directly or indirectly (Chapin et al., 2000; Hertenberger and Wanek, 2004) it can not be defined a priori how species number and / or species composition affect these soil variables. Therefore we can not state which soil or plant variables are represented by one of the four discriminant functions. However, there are a number of factors that are known to change in the course of altered plant diversity which therefore have the potential to alter the soil amino acid fingerprint: First, root exudates have been shown to be the main source of the soluble light molecular weight organic substances (LMWOS) pool in soil (Fischer et al., 2007) and plant species significantly differ in the composition of their root exudates (Marschner et al., 2004). Amino acids on the other hand are known to be one of the main constituents of root exudates (Bowen and Rovira, 1999; Nguyen, 2003; Jones et al., 2004) and Fischer et al. (2007) have shown that by this, plants affect the concentration and composition of free amino acids in soil. This might be one reason for our successful separation of the different plant diversity levels by means of discriminant analysis of the free amino acid fingerprint in soil. However, Fischer et al. (2007) also found evidence that the amino acid fingerprint is altered by the metabolic activity and composition of the soil microbial community. As most soil microbes are heterotrophic using root exudates and plant debris as food it has to be assumed that any change in the composition of this C-input as induced by altered plant diversity leads to a change of composition or activity of the microbial soil community (Korthals et al., 2001; Liu et al., 2008). This reveals that any change in the soil amino acid fingerprint is initiated by alterations of plant diversity and composition but is also affected by a number of additional processes like microbial degradation which themselves are influenced by plant diversity. It is plausible that these complex interactions and feedback mechanisms are mirrored by the need of more than a single discriminant function to separate groups of different diversity levels. However, the discriminant analysis enabled us to identify groups differing in diversity that are similar with respect to one process or a combination of processes leading to a characteristic amino acid fingerprint, which was the primary aim of our investigation.

4 Conclusions

We were able to show that despite an increasing plant productivity with increasing plant diversity, amino acid concentrations in soil did not change significantly. Results in literature suggest that the insignificant increase of amino acid

concentrations might be caused with an increase of size and activity of the microbial community.

However, the fingerprint of free soil amino acids changed significantly between plant diversity levels. In this we showed that grassland plots representing different plant diversity levels can be separated successfully by means of discriminant analysis. Our experimental design did not allow for any functional characterisation of the single discriminant functions. However, a cross search in literature showed that the soil amino acid fingerprint is influenced by the composition of root exudates and the composition and activity of the soil microbial community. Therefore the amino acid fingerprint represents a global parameter characterizing the system status with respect to C and N input and degradation. Discriminant functions that are capable to separate single groups therefore indicate that the fingerprint of these groups is controlled by similar processes.

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Study 5: Plant diversity affects the outcome of competition between plants and microbes for organic N

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Abstract

Uptake of amino acids is a well described potential of plants opening up a new possibility for niching with respect to N-acquisition. Though niching or source partitioning are important models to explain the positive plant diversity-productivity relationship in diversity experiments, knowledge about the use of amino acids as an alternative N-source for plants in these experiments is scarce.

We therefore conducted a tracer study applying dual labelled (^{13}C, ^{15}N) amino acids to plots of a grassland diversity experiment. Diversity was altered from 1 to 16 plant species containing 1 to 3 functional plant traits. Total tracer N uptake of the microbial soil community and plants was measured in bulk material 24 h after label application and in addition the uptake of intact amino acids by plants was measured via compound specific stable isotope measurements.

Most of the tracer N was taken up by microbes (40 – 80 %) while only 1.5 to 3.8 % were taken up by plants. Overall plants showed a decreasing N-uptake with increasing diversity and a mixed effect model showed that this was mainly due to the intense microbial competition for N. However, the uptake of intact amino acids by plants increased at the same time and thus the contribution of organic N to the plants total N uptake increased from 1.5 % for monocultures to 7.1 % for the 16 species mixtures. This change was mainly promoted by the plants N-concentration and their root density, i.e. plants with a low N-concentration and a high root density showed highest amino acid uptake. Despite the overall declining N-uptake of plants with increasing diversity, the plants productivity and the amount of N stored in the plant biomass increased at the same time. This indicates that the superior power of microbes for N uptake is true for the short term uptake but might be inverse in the long term. In this the increased N-storage in plant biomass is also promoted by the plants increased N-source partitioning, i.e. the more intense use of amino acids as alternative N-source.

Key words: organic N, plants, microbes, competition, diversity, amino acids

1 Introduction

Most biodiversity studies during the last two centuries reported a positive effect of biodiversity on plant productivity (Tilman et al., 1996; Hector et al., 1999; Loreau et al., 2001; Hooper et al., 2005; Spehn et al., 2005; van Ruijven and Berendse, 2005; Marquard et al., 2009), which was one of the main factors explaining the positive effect of diversity on increasing C-storage (Gleixner et al., 2005; Steinbeiss et al., 2008) and nutrient retention capacity in soil. Despite contrasting results from natural ecosystems showing no effect of diversity on productivity (Waide et al., 1999; Enquist and Niklas, 2001; Mittelbach et al., 2001), it is generally accepted that ecosystems differing in plant diversity but not in environmental conditions show a positive diversity effect on productivity (Loreau, 1998; Loreau et al., 2001; Schmid, 2002). However, the mechanisms enabling plants to be more productive in more diverse systems are still under debate. One possible mechanism is higher complementarity in resource use, i.e. the use of differing resource niches by different plant species (Tilman et al., 1997; Loreau, 1998) where niches can be defined on a temporal, spatial and chemical scale (Trenbath, 1974; Harper, 1977; Ewel, 1986; Vandermeer, 1989). From this, Hooper et al. (2005) inferred that only if the respective resources are growth limiting, complementary or niche separation will lead to a higher resource use efficiency and productivity. Apart from water, the most important growth limiting nutrient in soil is nitrogen (Vitousek and Howarth, 1991). This well established knowledge is also supported by the positive legume effect on biodiversity-productivity relationships (Hooper and Vitousek, 1997; Tilman et al., 1997; Symstad et al., 1998; Mulder et al., 2002; Spehn et al., 2005). But a positive biodiversity effect on plant productivity is not limited to the presence of legumes (van Ruijven and Berendse, 2003; Marquard et al., 2009) which indicates that additional niches in N-acquisition exist (Roscher et al., 2008; von Felten et al., 2009). It is long standing knowledge that plants have the physiological capacity to take up intact amino acids via their root system (Virtanen and Linkola, 1946) and this potential has been demonstrated for various plants in natural and agricultural ecosystems varying in soil type, soil temperature and moisture regimes (Stribley and Read, 1980; Jones and Darrah, 1994; Kielland, 1994; Yamagata and Ae, 1996; Näsholm et al., 1998; Schmidt and Stewart, 1999; Näsholm et al., 2001; Weigelt et al., 2005). Though there is no doubt that plants are able to use this organic N form under natural conditions, the ecological relevance of this process is controversially discussed (Lipson and Näsholm, 2001; Jones et al., 2005a). It was argued that microbes completely outcompete plants for the uptake of amino acids

(Black, 1993). Therefore, amino acid uptake might only be of importance in ecosystems, were microbial mineralization rates are generally low and the soil N pool is dominated by amino acids. Amino acid uptake therefore was first investigated in nitrogen limited ecosystems with low annual soil temperature like boreal forest and tundra (Näsholm et al., 1998; Näsholm and Persson, 2001; Nordin et al., 2001; Persson and Näsholm, 2001; Persson et al., 2003; Kielland et al., 2006), or alpine ecosystems (Raab et al., 1996; Lipson and Monson, 1998; Miller and Bowman, 2003; Xu et al., 2004; Xu et al., 2006). Here, McKane et al. (2002) demonstrated that in arctic tundra uptake of organic N was not only the most abundant form of N-nutrition, but that dominant plants also used the organic N as the most abundant N form in soil while subdominant plants take up less abundant forms. On the one hand this implies that in temperate ecosystems most of the N is derived from the dominant mineral N pool (Harrison et al., 2007) and as a consequence uptake of organic N will be of minor importance. On the other hand the results of McKane et al. (2002) also show, that N partitioning might enable subdominant plants to grow along with highly productive species and that the overall N uptake of the plant community will be higher. Higher plant productivity with increasing plant diversity might thus boost the importance of N partitioning or might even depend on the resulting higher N uptake.

We therefore investigated changes of direct plant amino acid uptake and its contribution to the plants' N-nutrition in a grassland diversity experiment. We used double labelled amino acids to measure the uptake of intact amino acids and mineral nitrogen derived from microbial tracer mineralization. Rasmussen et al. (2009) suggested that amino acid uptake might be biased by the uptake of inorganic tracer derived carbon and Sauheitl et al. (2009b) showed that it is not possible to distinguish this uptake from direct amino acid by isotope bulk measurements of plant tissues. Therefore we used compound specific isotope measurements which were shown to be a more accurate tool for the investigation of direct amino acid uptake (Sauheitl et al., 2009b). As a novel approach in amino acid uptake studies, we calculated the effect of tracer dilution by natural soil-N pools as suggested by Buchmann et al. (1995). This enabled us to calculate the plants' total amino acid uptake from soil (tracer + soil amino acids) which is a more meaningful measure than pure tracer uptake with respect to the ecological impact of amino acid uptake. Due to a positive diversity-productivity effect in the investigated grassland we assume that:

(1) Plant total N as well as direct amino acid uptake will increase with increasing

diversity.

(2) N partitioning will be intensified and as a result the contribution of amino acid N to the plants N nutrition will increase.

(3) Enhanced N partitioning is caused by enhanced N competition between plants and microbes.

2 Material and Methods

2.1 Study area

The experiment was carried out in August 2007 on plots of the *Jena Experiment* (Roscher et al., 2004), which is located on a flood-plain of the Saale river near Jena, Germany (11°34'60" East; 50°55'60" North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was highly fertilized during the last 40 years of agricultural utilisation until 2002 when the *Jena Experiment* started. Labelling was done on 28 selected plots of 3.5 x 3.5 m size (small replicates of main plots, see Roscher et al. (2004) for details on the experimental design). Plots were established from seeds using a pool of 48 common Central European grassland species that had been assigned to three functional groups: 16 grasses, 12 small herbs and 20 tall herbs. The original design of the Jena-Experiment includes 12 legumes, but plots with legumes were omitted for the current analysis. Plots represented a biodiversity gradient from 1, 2, 4, 8 and 16 species with 6 plots per diversity level except for the 16-species mixtures where only 4 plots were used. One year before the experiment started, one individual of each of three phytometer species (*Plantago lanceolata, Geranium pratense* and *Festuca pratense)* were planted in the center of three rings located in one quarter of each plot (Figure 5.1). This was done as it had been shown in literature that the uptake potential for amino acids of different plant species widely varies (Weigelt et al., 2003; Weigelt et al., 2005). Changes in the amino acid uptake between plots of different biodiversity might therefore be rather due to changes in sampled species than due to the effect of altered biodiversity. The sampling of plant species that are present in all plots (phytometers) removes this effect, leaving plant diversity as the sole manipulated variable.

Four weeks before labelling started each plot was equipped with polyethylene tubes (PE) of 30 cm diameter that allowed to measure gas efflux from the soil (rings in Fig. 5.1). The tubes were installed to a depth of 30 cm leaving a ring of 10 cm height above-

ground on which a gas tight top could be fixed for gas measurements. Each ring enclosed one phytometer cluster in its centre.

One week before tracer application, total vegetation cover (%) was estimated visually in all rings.

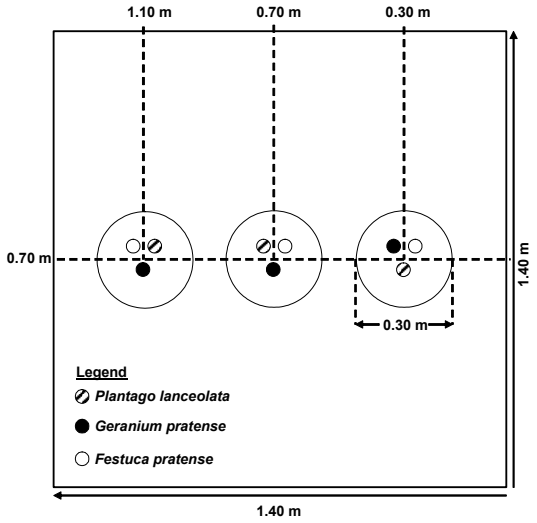

Figure 5.1: Sketch of a field plot with gas sampling rings, each containing three phytometers.

2.2 Labelling

One of the three rings with phytometer clusters of each plot was used for measurements of natural isotopic composition of plant and soil material, while the other two received a solution of dual labelled (^{13}C and ^{15}N) amino acids. The used amino acid solutions contained equal amounts of glycine, valine and phenylalanine either in a labelled form (98 at% ^{13}C and ^{15}N) or in an unlabelled form for application in the background rings. The respective solutions were injected into the soil to 5 cm soil depth using a dispenser (Eppendorf multipette® 4780, Eppendorf, Hamburg, Germany) that was connected via a silicon tubular to a stainless steel needle with a side hole. Funnels were placed round the injection needle to impede contamination of above ground biomass. To avoid clogging of the syringe holes were drilled into the soil using a small screwdriver before tracer application. 17 injections of 2 mL were applied in a radiating pattern within each ring to ensured a homogenous dispersion of the solution within the PE tubes. This resulted in a total volume of 34 mL containing app. 130 µmol of each amino acid. The two labelled rings differed in sampling time, i.e. while one ring was harvested completely after 24 hours (short term experiment) the other ring was sampled on five consecutive time steps within four weeks after application (long term experiment). As it has been shown that intact amino acid uptake can best be measured within a short time after tracer application (Streeter et al., 2000) the measurements of amino acid uptake

were only done in the short term rings while the long term rings were used to investigate the fate of tracer derived ^{13}C and ^{15}N in soil and soil gas efflux over a longer time span. However, we will only present the results of the short term rings here while the data on the long term turnover of amino acid tracer will be published elsewhere (Barnard et al., in preparation).

2.3 Sampling

Exactly 24 h after labelling, above ground plant biomass was cut at soil level and sorted into phytometer species and plot species. Immediately afterwards, four soil samples were taken to a depth of 30 cm using a metal corer. One soil sample was taken below each of the three phytometers, respectively, and the fourth sample was located in the centre between the phytometers. Soil and plant samples were immediately cooled in a cooling box and brought to the lab within two hours. There, plant samples were frozen in liquid N_2 and stored at -40 °C until further analysis. Soil samples were sieved to < 2 mm, roots collected and washed according to Sauheitl et al. (2009a). After washing, roots were frozen in liquid N_2 and stored at -40 °C until further analysis. Residual soil was also frozen in liquid N_2 and stored at -40 °C until further analysis except for 50 g of sieved soil that were stored at 5 °C in a refrigerator for successive extraction of microbial biomass.

2.4 Amino acid extraction from plant material

Plant material was dried using a freeze dryer (Christ Beta 1-8, Christ GmbH, Osterode, Germany) and grinded to a fine powder. All free and protein bound amino acids in shoot and root tissues were thereafter extracted using hot acidic hydrolysis as described in Amelung et al. (2001). The following purification of the hydrolysates and derivatization of the hydrolyzed amino acids was done as outlined by Amelung et al (2001) and Sauheitl et al. (2009b). The resulting solution containing the amino acid derivatives was transferred to gas chromatographic vials and was analyzed immediately with an isotope mass spectrometer system (IRMS) as described below.

2.5 Extraction of free amino acids in soil

Free amino acids in soil were extracted following the procedure of Houba et al. (1986) and Mengel et al. (1999) with small changes in extraction time and use of internal standards as outlined in Sauheitl et al. (2009, in preparation). In short, app. 15 g of dry sieved soil were extracted with 100 mL of a 0.01 M $CaCl_2$ solution at 80 °C. Soil and extraction solution were shaken for 25 min. and the soil was separated by centrifu-

gation at 2000 rpm followed by transferring of the supernatant to conical flasks. There the solution was concentrated to app. 2 mL using a rotary evaporator and 200 µL of the final solution were purified and derivatized using the amino acid analysis kit EZ:faast® (Phenomenex, Torrance, CA, USA). The purification and derivatization procedure as used for plant amino acids had to be exchanged by the EZ:faast® procedure due to high cation concentrations in soil eluates that disturbed the derivatization procedure by Amelung et al. (2001). The instructions for the procedure were provided by the manufacturer and are explained in detail elsewhere (Kugler et al., 2006).

2.6 Extraction of microbial biomass

The extraction of microbial biomass from soil was done one week after sampling had been finished. The general procedure followed the method by Brookes et al. (1985) with slight modifications: 10 – 25 g of soil were filled into plastic cups, adjusted to app. 50 mass% water content by addition of deionised water an placed in a dessicator containing a glass, filled with alcohol free $CHCl_3$. Vacuum was generated in the dessicator and soil samples were fumigated for 24 h. A second batch of the same samples stayed unfumigated and both were extracted with 0.03 M K_2SO_4 for 30 min. on a horizontal shaker at maximum speed. The soil in the extract was allowed to sediment and the supernatant was filtered (Schleicher & Schüll 589, 150 mm) into plastic tubes, frozen and freeze dried before measurement on an elemental analyser (EA) that was coupled to an IRMS.

The concentration of the K_2SO_4 solution was reduced from 0.05 M as used by Brookes et al. (1985) to 0.03 M as high salt concentration in the dried eluates were shown to cause problems when measuring ^{15}N and ^{13}C enrichment on an EA-IRMS. However, as we used the extraction coefficient introduced by Brookes et al. (1985) for the calculation of microbial N and C amount, we processed a second batch of samples using 0.05 M K_2SO_4. These samples were also frozen, stored at -30 °C and used for analysis of microbial C and N amount on a liquid CN analyser.

2.7 Substance specific isotope measurements of plant material

Intact amino acid uptake by plants was calculated using substance specific isotope measurements. Compared to the conventional bulk measurements this assures that only the factual uptake of intact amino acids but no additional uptake of tracer fragments is measured (Sauheitl et al., 2009b). The ^{13}C signature of protein bound amino acids in plant tissues and of free amino acids in soil was measured using a GC (Trace

GC 2000, Thermo Finnigan) coupled to an IRMS (Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany) via a combustion interface (GC-C-IRMS). Detailed info on the instrument setup are given in Sauheitl et al. (2005) and GC settings for the peak separation of protein bound amino acids are outlined in Sauheitl et al. (2009b). GC settings changed for measurements of free amino acids, as a different derivatization was used: 2 µL of the sample solution were injected into the injector of the GC at a temperature of 250 °C. A Zebron ZB-AAA GC-column (10 m x 250 µm x 10 µm, Phenomenex) was used for peak separation and column flow was set to 2.0 mL for 7 min. decreased by 1.0 mL*min.$^{-1}$ to 1.5 mL and kept constant for 18.38 min. The temperature of the GC oven was programmed to 90 °C for 1 min. raised to 155 C at a rate of 5.0 °C*min^{-1} raised to 240 °C at a rate of 10 °C*min.$^{-1}$ and raised to 320 °C at a rate of 120 °C*min^{-1}.

Online calibration of $\delta^{13}C$ values was done by the injection of CO_2 reference gas (99.7 % purity) into the IRMS during measurements. All δ-values were referenced to international isotope standards (Glaser and Amelung, 2002; Sauheitl et al., 2009b).

2.8 Bulk measurements of plant material

The $\delta^{15}N$ values of root and shoot tissues were measured on an EA (Carlo Erba NC 2500, Thermo Finnigan) coupled to an IRMS (Delta Plus^{TM}, Thermo Finnigan). For calibration of $\delta^{15}N$ two types of $(NH_4)_2SO_4$ (IAEA-N1, $\delta^{15}N$ = 0.36 ‰ and IAEA-N2, $\delta^{15}N$ = 20.3 ‰), KNO_3 (IAEA-N3, $\delta^{15}N$ = 4.7 ‰) and two ^{15}N-enriched forms of $(NH_4)_2SO_4$ (USG-26, IAEA, $\delta^{15}N$ = 53.7 ‰ and USG-32, IAEA, $\delta^{15}N$ = 180 ‰) were used.

2.9 Analysis of microbial C and N

The amount of microbial C and N in the fumigated and unfumigated soils was measured on a liquid total carbon / nitrogen analyser (DIMA TOC-100, Dimatec, Essen, Germany).

All bulk ^{13}C and ^{15}N measurements were performed on an EA-IRMS (Delta^{plus}XP, Finnigan) using international standards Caf-z1 and Ali-z2 for referencing.

3 Calculations and Statistics

3.1 Calculation of tracer uptake

All calculations on isotope measurements were done using the at% notation. Therefore the measured δ-values were transformed to at% according to Craig (1953).

As the measured amino acids were derivatives, they contained additional C from the derivatization agent which changes their natural isotopic composition. This effect was therefore calculated and removed according to Glaser et al. (2002). Sauheitl et al. (2009b) has shown that the ^{13}C enrichment of plant amino acids, that were applied as tracer, reflects the plant uptake of intact tracer amino acids. If tracer is taken up by the plant, the isotopic signature of the plant's amino acids will change according to a two pool mixing model (Gearing, 1991) and the amount of tracer uptake can be calculated (Equ. 1).

$$A_{T,X} = \frac{\text{heavy isotope}_0 \cdot A_0 - R_{sample} \cdot \text{light isotope}_0 \cdot A_0}{R_{sample} \cdot \text{light isotope}_T - \text{heavy isotope}_T} \quad (1)$$

With A_0 representing the amount of plant amino acid C in mol and *heavy / light isotope*$_0$ giving its concentration of heavy and light isotope before tracer application in at%. $A_{T,X}$ gives the amount of tracer C taken up into the plant in the form of an amino acid *x* and its isotope composition of heavy and light isotopes both in at%. R_{sample} finally gives the ratio of heavy to light isotope in the sample. We thereafter calculated the sum of tracer uptake for all three amino acids and will further refer to this as *intact tracer uptake*, depicted in µmol C. As tracer contained heavy and light isotopes, we calculated the uptake of total tracer C (^{13}C + ^{12}C) and N (^{15}N + ^{14}N), respectively.

The amount of tracer N taken up in the form of amino acids is calculated by multiplication of $A_{T,X}$ with the C : N ratio of the respective amino acid. This was done as Sauheitl et al. (2009b) found that measured ^{15}N enrichment in amino acids overestimates intact N uptake due to the transfer of tracer derived N to newly formed amino acids. This will further on be denoted as *intact tracer N uptake* and as *total intact tracer N uptake* for the sum of intact tracer N uptake of all tracer amino acids.

Equation (1) was also used to calculate the overall uptake of tracer N into plants (in form of amino acids plus mineral nitrogen) and microbes, where A_0 is replaced by plant and microbial N amount and all following variables are replaced by the bulk ^{15}N (*heavy isotope*), ^{14}N (*light isotope*) and R values of microbial biomass and plant tissues, respectively. For microbes the amount of tracer uptake was calculated separately for fumigated and unfumigated samples using the respective N amounts and N isotope values in equation (1). In this the measured N amounts were corrected for extraction efficiency by the extraction factor 1/0.45 as suggested by Brookes et al. (1985). The total amount of tracer uptake by microbes was then calculated as the difference between tracer uptake in fumigated and unfumigated samples. The resulting tracer uptake will be

denoted as *total tracer N uptake* for microbes and plants, respectively. The same was done to calculate the total tracer derived ^{13}C uptake for microbes, termed as *total tracer C uptake* of microbes.

In addition we calculated the amount of tracer derived N taken up by plants in a mineral form as the difference between *total plant tracer N uptake* and *plant intact tracer N uptake,* denoted as *plant tracer N_{min} uptake*.

As the applied ^{15}N and ^{13}C was diluted by natural soil amino acids and mineral N, both already present before tracer application, the two tracer uptake rates of plants were corrected for this dilution effect. This was done by calculating the ^{13}C and ^{15}N signature of plant available soil amino acids and mineral N after the application of tracer which also followed a two component mixing system as shown in equation (1). To do so, equation (1) was solved for R_{sample} which now represents the isotope ratio of the respective substance (amino acids or mineral N) in soil after tracer application, denoted as $R_{soil,\ applied}$ (equation (2)).

$$R_{soil,\ applied} = \frac{\text{heavy isotope}_0 + \text{heavy isotope}_T \cdot A_{T,\ X}}{\text{light isotope}_T - \text{light}_0 \cdot A_0} \qquad (2)$$

All variables are specified as in equation (1) except for the fact that the investigated compartment is soil instead of plant tissues. The resulting isotope ratio was then used to calculate the at% values of light and heavy isotope in the respective substance according to equation (3):

$$R = \frac{\text{light isotope}}{\text{heavy isotope}} \qquad (3)$$

The resulting at% values were then placed in equation (1) to calculate the total uptake of nitrogen and amino acids (tracer derived plus soil derived), further on denoted as *total amino acid* or total *nitrogen uptake* of plants.

We will use tracer uptake values (as derived from equation (1)) to describe the competitive status between microbes and plants with respect to the added amino acid tracer, acting as a model substance. The ecological impact of amino acid uptake for the plants N nutrition will be investigated using the plants' total uptake values of N and amino acid N which include tracer and natural soil N uptake.

A list of all measured and calculated variables and their abbreviations is given in Table 5.1.

Table 5.1: Overview of all measured and calculated variables and abbreviations as used in the text.

Measured variables

Variable	Compartment	Abbreviation	Type of measurement
Plant Cover [%]	Aboveground Plants	*Cover*	visual estimation in field
Biomass [g]	All plant tissues	-	weighing
Soil density [g*cm^{-3}]	Soil	-	weighing
Gravimetric water content [%]	Soil	*grav. water content*	weighing
N amount [µmol]	Plant tissues, microbial biomass, mineral N in soil	-	calibrated instrument measurement
C amount [µmol]	Plant tissues, microbial biomass	-	calibrated instrument measurement
Amino acid amount [µmol]	Plant tissues, soil	-	calibrated instrument measurement
^{13}C content bulk [at%]	Plant tissues, microbial biomass	-	calibrated instrument measurement
^{15}N content bulk [at%]	Plant tissues, microbial biomass, mineral N in soil	-	calibrated instrument measurement
^{13}C in amino acids	Plant tissues, soil[1]	-	calibrated instrument measurement

Calculated variables

Variable	Compartment	Abbreviation	Used measured variables
Tracer N uptake	Plant tissues, microbial biomass	*total plant / microbial tracer N uptake*	*N plant* , *N mic* , ^{15}N plant, ^{15}N mic,
Tracer C uptake	microbial biomass	*total tracer C uptake*	*C mic,* ^{13} *C mic*
Tracer amino acid uptake	Plant tissues	*intact tracer uptake*	AA, ^{13}C AA
Total tracer amino acid uptake	Plant tissues	*total intact tracer uptake*	*intact tracer uptake*
Tracer amino acid-N uptake	Plant tissues	*intact tracer-N uptake*	*intact tracer uptake*
Total tracer amino acid-N uptake	Plant tissues	*total intact tracer-N uptake*	*intact tracer-N uptake*
Mineral tracer N uptake	Plant tissues	*plant tracer N $_{min}$ uptake*	*total plant tracer N uptake, total intact tracer-N uptake*
Amino acid uptake	Plant tissues	*amino acid uptake*	AA_{plant}, AA_{soil}, ^{13}C AA, ^{13}C AA_{soil}
Total amino acid uptake	Plant tissues	*tot. amino acid uptake*	*amino acid uptake*
Amino acid-N uptake	Plant tissues	*tot. amino acid-N uptake*	*Tot. amino acid uptake*
Mineral N uptake	Plant tissues	*plant N $_{min}$ uptake*	*N plant* , ^{15}N plant, $N_{min,soil}$
Total N uptake	Plant tissues	*total N uptake*	*tot. amino acid-N uptake, plant N $_{min}$ uptake*
Contribution of amino acid N to the total N uptake of plants	Plant tissues	*AA-N in total N uptake*	*Tot. amino acid uptake, total N uptake*

[1]: Only measured in background soil samples

3.2 Statistics

All measured variables were tested for outliners using the Nalimov test (Gottwald, 2000). Further statistics were performed using *SPSS for Windows* (Vers. 10.0.1, SPSS GmbH, Munich, Germany) and *Statistika* (Vers. 6.0, Statsoft GmbH,

Hamburg). All data were tested to be normally distributed and variances between groups to be equal using the Shapiro-Wilk and the Levene test, respectively. The influence of species richness on these variables was investigated using the analysis of variance (ANOVA) procedure followed by a Sheffé post-hoc test. If data were not normally distributed or variances were not equal, a non parametric Kruskal-Wallis ANOVA was conducted, followed by a Games-Howell post-hoc test. To elucidate links and correlations between plant, microbial and soil parameters, general linear models (GLM) were adjusted to the data with microbial and plant biomass and microbial and plant tracer uptake as dependent variables. As there was a gradient in soil parameters like texture and N concentration throughout the field site, the field has been organised in a four block design (Roscher et al., 2004) and plot replicates of the same species richness were randomly distributed on these blocks. To account for this, the block effect was implemented in the GLM as a random effect and the GLM was conducted as a mixed effect model using Sum of Square Model III for fitting.

4 Results

4.1 Plant biomass

The aboveground biomass of the plant community (sown species) of each labelled ring showed a rising trend with increased species richness (ANOVA, $p < 0.05$) (Table 5.2). From monocultures to 8 species mixtures the average above ground biomass nearly tripled. However, this was no continuous trend as a post-hoc test revealed that plots with species richness (SR) 8 showed highest biomass values, while this variable decreased again for the 16 species mixtures.

The N concentration in the plant community biomass decreased significantly (ANOVA, $p < 0.05$) from 1.25 % for SR1 to 0.77 % for SR16 (Table 5.2). This trend was not consistent from SR1 to SR2 and from SR4 to SR8 which did not differ in N concentration. Total N storage showed an increasing trend (ANOVA, $p < 0.05$) with increasing diversity (Table 5.2), with lowest amounts (180 mg N $ring^{-1}$) for monocultures and highest amounts (250 mg N $ring^{-1}$) for SR4.

4.2 Mineral soil N

The nitrate concentrations in soil before tracer application were frequently below the detection limit of the CFE measurement. We therefore will only present values on ammonium here as this was the dominant mineral N species. Ammonium concentra-

tions varied from 0.8 to 1.2 µg N g^{-1} dry soil (Table 5.2) and were significantly positively influenced by species richness (ANOVA, $p < 0.05$).

Table 5.2: Means ± standard errors with increasing species richness levels (SR) and the results of a mixed ANOVA with significant effects given in bolt.

Variable	SR1	SR2	SR4	SR8	SR16	Effect	F	p
shoot biomass [g]	5.72 ± 1.33	9.58 ± 1.78	13.22 ± 2.17	17.31 ± 4.51	14.11 ± 2.95	SR	3.747	**0.022**
						Block	1.199	0.338
N conc. plant [%]	1.25 ± 0.06	1.16 ± 0.09	1.02 ± 0.03	1.04 ± 0.04	0.77 ± 0.17	SR	3.329	**0.033**
						Block	0.886	0.467
N plant*ring^{-1} [mg]	177.98 ± 18.19	214.51 ± 22.36	246.57 ± 28.41	237.24 ± 36.93	236.16 ± 29.45	SR	1.536	0.234
						Block	1.577	0.230
N_{min} soil [µg*g soil^{-1}]	0.84 ± 0.08	0.89 ± 0.07	0.82 ± 0.06	0.96 ± 0.08	1.17 ± 0.23	SR	3.232	**0.036**
						Block	2.877	0.065
C mic [µmol C*g soil^{-1}]	46.24 ± 2.01	52.13 ± 2.70	54.91 ± 2.01	58.24 ± 3.21	52.95 ± 7.40	SR	5.783	**0.004**
						Block	4.419	**0.017**
C/N mic	7.04 ± 0.08	7.29 ± 0.20	7.40 ± 0.13	7.62 ± 0.08	7.40 ± 0.30	SR	1.442	0.305
						Block	1.531	0.277
mic. tot. tracer C uptake [µmol C]	163.08 ± 33.15	26.13 ± 31.31	152.1 ± 21.51	317.38 ± 41.14	189.04 ± 45.38	SR	1.954	0.145
						Block	2.952	0.060
mic. tot. tracer N uptake [µmol N]	0.16 ± 0.03	0.23 ± 0.03	0.15 ± 0.02	0.32 ± 0.04	0.19 ± 0.05	SR	1.37	0.284
						Block	1.526	0.242
plant tot. tracer N uptake [µmol N]	14.41 ± 2.69	12.45 ± 2.97	9.59 ± 1.38	9.09 ± 1.06	5.89 ± 0.62	SR	2.691	**0.049**
						Block	0.155	0.920
plant tot. intact tracer uptake [µmol C]	1.48 ± 0.29	2.48 ± 0.3	2.54 ± 0.29	2.02 ± 0.27	1.72 ± 0.26	SR	3.701	**0.023**
						Block	1.452	0.261
plant tot. N uptake [µmol N]	63.45 ± 10.64	36.48 ± 6.75	40.04 ± 2.39	31.16 ± 4.75	22.04 ± 3.52	SR	4.045	**0.016**
						Block	0.555	0.651
plant tot. amino acid uptake [µmol N]	6.7 ± 0.86	10.76 ± 1.74	10.84 ± 0.87	9.27 ± 1.24	13.33 ± 1.76	SR	4.579	**0.010**
						Block	2.16	0.128
AA-N in total N-uptake [%]	1.47 ± 0.08	5.15 ± 0.92	4.15 ± 0.32	4.63 ± 0.64	7.04 ± 0.26	SR	14.997	**< 0.001**
						Block	1.38	0.281

4.3 Microbial biomass

Microbial biomass content in soil depicted as microbial C per g dry soil showed a positive trend with increasing species richness (Table 5.2, ANOVA, $p < 0.05$). Microbial biomass increased from 46 to 58 µmol C g^{-1} dry soil between SR1 and SR8. Though the C : N ratio of the microbial biomass increased from 7.04 at SR1 to 7.62 at SR8, this trend was not significant (Table 5.2; ANOVA, $p < 0.05$). The mixed effect model on microbial biomass could explain 94.71 % of the observed variations of microbial C content (Table 5.1_{supp} in supplementary). The variables with the highest explanatory power where block (32 %) and plant species richness (10 %). The final function of the adjusted model had an R^2 of 0.95.

4.4 Microbial tracer uptake

The deviation of microbial uptake of tracer derived nitrogen and carbon was equal for both elements (Fig. 5.2) resulting in a linear correlation between both uptake variables (R^2=0.8327, $p < 0.001$). There was no consistent trend of tracer uptake alteration with increasing species richness and tracer C and N uptake were not significantly influenced by plant species richness. Plots of SR8 showed highest microbial tracer N and C uptake while all other treatments did not differ significantly from one another. The range of tracer uptake was 0.651 – 1.416 mmol for carbon and 0.151 – 0.317 mmol for N, i.e. 32 – 69 % and 39 – 81 % of the applied tracer C and N were taken up by the microbial community, respectively. In average 48 % and 54 % of applied tracer C and N were incorporated into the microbial biomass, respectively. The mixed effect models explained 99.46 and 97.90 % of the variability in microbial total tracer C (Table 5.2_{supp} in supplementary) and N (Table 5.3) uptake with an R^2 of 0.99 and 0.98, respectively. For both dependent variables the highest proportion of the overall variance was explained by the combination of functional plant groups and the block effect (52.5 % for C uptake, 48.5 % for N uptake), followed by shoot biomass (7.3 and 4.9 %). However, the used regression coefficients for the latter one were very small (-0.002 and -0.001) and impact of this variable on tracer uptake therefore was inferior.

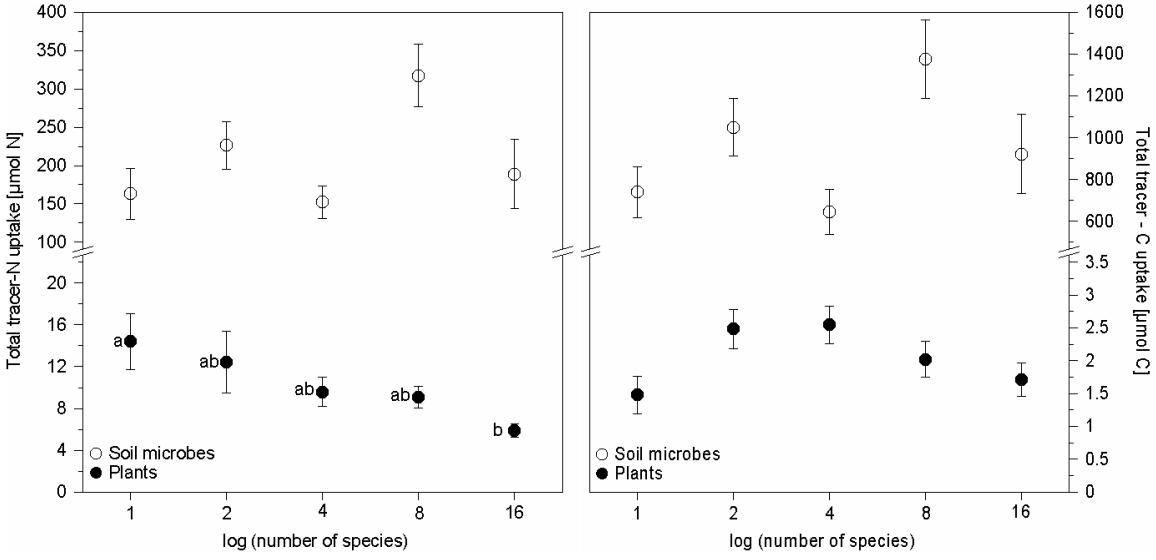

Figure 5.2: Total tracer N (left panel) and total tracer C (right panel) uptake by soil microbes (filled circles) and plants (open circles). Given are means ± standard errors per ring for plots with increasing plant species richness.

Table 5.3: Mixed effect model on *total microbial tracer N uptake*; SR gives species richness, fgc depicts combination of functional plant groups and BLOCK gives the block effect.

Source	% Var. explained	Reg. Coeffizient	df	F	Sig.
Intercept	2.003	7.05	1	3.73	0.125
microbial C	3.258	0.04	1	6.08	0.069
shoot biomass	4.911	-0.02	1	9.16	0.039
pH	2.07	-1.07	1	3.86	0.121
SR	1.425	-0.07	1	2.66	0.178
fct. group comb. (fgc)	20.23		6	0.72	0.646
BLOCK	11.48		3	1.50	0.262
fgc * BLOCK	52.48		10	9.79	0.021
Total explained	97.86				
Total error	2.145				

$R^2 = 0.98$

4.5 Plant tracer uptake

Total uptake of tracer derived nitrogen included the uptake of N in the form of intact tracer molecules and mineral N as derived from tracer mineralization. In contrast to total plant nitrogen uptake, this does not include the uptake of soil N and soil amino acids. The same applies to the difference between total uptake of intact tracer amino acids and the total uptake of amino acids.

Total tracer N uptake of plants declined significantly with increasing species richness (ANOVA, $p < 0.05$) (Fig. 5.2, Table 5.2) from 14.41 µmol N for SR1 to 5.89 µmol N for SR16 which was the lowest uptake value of all diversity levels ($p < 0.05$). Based on the applied tracer N amount this is equal to 3.75 % - 1.53 % tracer uptake for SR1 and SR16, respectively and results in an average uptake of 2.68 %. Therewith the plants' tracer N uptake was app. 15 to 30 times lower than that of the microbial biomass. A mixed effect model fitted to these data explained 78 % of the observed variations (Table 5.3_{supp} in supplementary), in which 18 % were explained by species richness and block effect, followed by microbial parameters (microbial abundance, microbial tracer C and N uptake) explaining 20 %, while plant parameters (root density, shoot biomass, N concentration) only accounted for 9 %.

In contrast to total tracer N uptake, the devolution of total uptake of intact tracer amino acids showed a different picture (Fig. 5.2). Lowest uptake rates were found for monocultures (1.5 µmol C) rising to 2.5 µmol in the case of SR2 and SR4 and finally falling back to 2.0 and 1.7 µmol C for SR8 and SR16. This is equivalent to a relative

uptake of 0.12 to 0.07 % of the applied tracer and was significantly influenced by species richness (ANOVA, $p < 0.05$). Again, these uptake rates were significantly (ANOVA, $p < 0.01$) smaller than those of the microbial biomass.

4.6 Total plant nitrogen and amino acid uptake

The calculated total uptake values for nitrogen and amino acids reflect the joint uptake of tracer and natural soil N and soil amino acids, respectively. Just as for tracer derived nitrogen uptake, *tot. nitrogen uptake* of plants declined with increasing species richness (ANOVA, $p < 0.05$) (Fig. 5.3, Table 5.2), at which absolute uptake values were significantly higher as those of *tot. tracer N uptake*. Plots of SR1 had highest uptake values (63 µmol N) decreasing to 36, 40, 31 and 22 µmol N for SR2, SR4, SR8 and SR16 in which SR16 revealed lowest uptake values ($p < 0.05$). The mixed effect model (Table 5.4A) revealed that most of the variance in *tot. nitrogen uptake* was explained by the species richness and block effect (21.8 %) followed by the three microbial parameters, microbial tracer-C uptake (10.8 %), microbial tracer-N uptake (7.8 %) and microbial carbon in soil (6.8 %). While gravimetric water content had a little but still significant explanatory power (5.2 %), all plant parameters as well as soil density were of minor importance (< 1 %). In total the model explained 81.43 % of the observed variance and the regression between observed and predicted N uptake values was highly significant ($p < 0.01$) with an adjusted R^2 of 1.00. Microbial soil carbon and microbial *tracer N uptake* had a negative effect on plant N-uptake, while the microbial *tracer C uptake* had a positive one.

Compared to the intact uptake of tracer amino acids by plants, the devolution of *tot. amino acid uptake* with increasing diversity slightly differed (Fig. 5.3). *Tot. amino acid uptake* increased significantly (Table 5.2, ANOVA, $p < 0.05$) from 6.70 to 10.76, 10.84, 9.27 and 13.32 µmol C for SR1, SR2, SR4, SR8 and SR16, respectively. Thus the total amino acid uptake doubled from monocultures to 16 species mixtures, although this trend was not found between 2, 4 and 8 species mixtures which did not differ to one another (post-hoc, $p < 0.05$). The fitted mixed effect model (Table 5.4B) explained 76.67 % of the observed variations in the data on *tot. amino acid uptake* by plants. Species richness and block effect explained most of the variance (13.35 %). The abiotic factors soil density and gravimetric water content explained 9.23 and 5.22 %, respectively. A smaller part was explained by plant N concentration (8.32 %) root density (7.59 %) and above ground biomass (4.64 %). While plant variables therefore explained 21 % of the total variance, the influence of microbial competition as indicated by tracer-

C uptake was small and insignificant ($p = 0.88$). While root density and aboveground biomass had a positive effect on amino acid uptake, the plants' *N-concentration* had a negative effect.

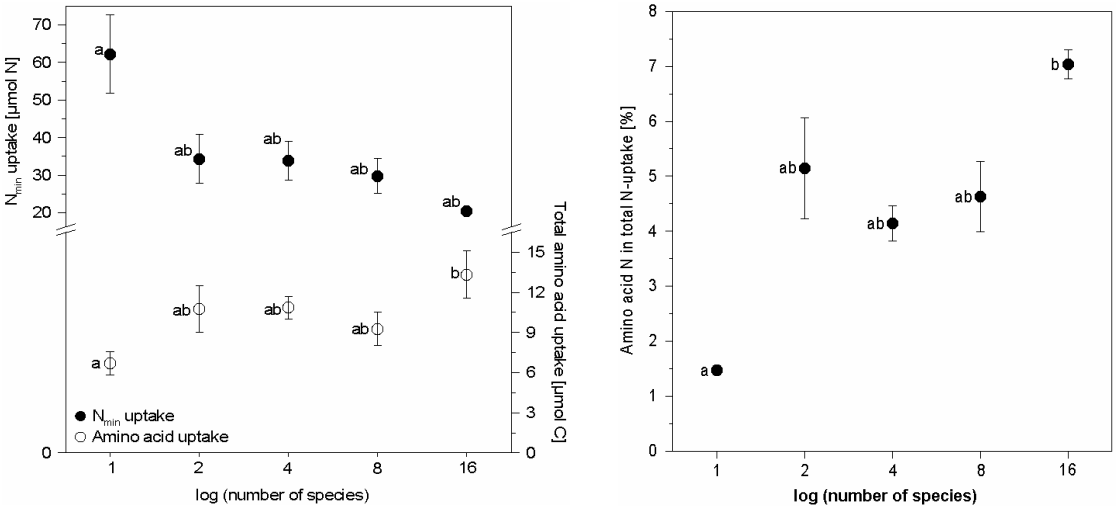

Figure 5.3: Plants total N (filled circles, left panel) and total amino acid uptake (open circles, left panel) and the percentage contribution of amino acid uptake to the plants total N uptake (right panel). Given are means ± standard errors per ring for plots with increasing plant species richness.

As a result of decreasing *tot. N uptake* and rising amino acid uptake with increasing species richness, the contribution of N taken up in the form of amino acids to the plants total N uptake increased significantly with increasing species richness (Table 5.2; Fig. 5.3; ANOVA, $p < 0.05$). The value rose from 1.47 % for SR1 to 5.14, 4.14, 4.63 and 7.04 % for SR2, SR4, SR8 and SR16, respectively.

Table 5.4: Mixed effect model on *total plant N uptake* (A) and on *total plant amino acid uptake* (B); SR shows plant species richness and BLOCK showes the block-effect. Arrows indicate a positive (↑) or negative (↓) effect.

A)

Source	% Var. explained	df	F	Sig.	
Intercept	5.36	1	5855.54	0.001	
microbial C	6.8	1	512873.95	0.001	↓
microbial C-uptake	10.83	1	806268.51	0.001	↑
microbial N-uptake	7.80	1	580897.99	0.001	↓
root density	0.31	1	23406.31	0.004	↑
shoot biomas	0.23	1	16808.30	0.005	↑
plant N-concentration	0.83	1	62076.60	0.003	↑
grav. water content	5.19	1	386110.58	0.001	↓
soil density	0.02	1	1525.36	0.016	↓
SR	16.80	4	2.54	0.133	
BLOCK	5.35	3	1.78	0.238	
SR * BLOCK	21.81	7	231867.09	0.002	
Total explained	81.43				
Total error	18.57				

$R^2 = 1.00$

B)

Source	% Var. explained	df	F	Sig.	
Intercept	9.71	1	52.60	0.005	
microbial C-uptake	0.01	1	0.03	0.879	
root density	7.59	1	41.20	0.008	↑
shoot biomas	4.64	1	25.21	0.015	↑
plant N-concentration	8.32	1	45.17	0.007	↓
grav. water content	5.22	1	28.34	0.013	↓
soil density	9.23	1	50.14	0.006	↓
SR	8.70	4	1.69	0.248	
BLOCK	9.92	3	2.99	0.095	
SR * BLOCK	13.35	7	10.36	0.041	
Total explained	76.67				
Total error	23.33				

$R^2 = 0.904$

5 Discussion

5.1 Plant and microbial biomass

Increasing plant species richness had a positive effect on above ground biomass in our experiment. The aspect of plant community productivity has been one of the first and still is one of the central properties investigated in studies on plant diversity. A number of studies investigating the effect of artificially altered plant diversity (Balvanera et al., 2006; Cardinale et al., 2006; Marquard et al., 2009) or natural plant diversity (Flombaum and Sala, 2008; Tylianakis et al., 2008) on plant productivity also found this positive correlation. In addition, we found a significant decrease in plant N-concentration with increasing plant diversity which could either be explained by plant internal factors reducing N uptake (root uptake surface, shoot biomass as sink, enzyme activities etc.) or by soil borne factors reducing N-availability such as increased microbial competition. The potential of roots to take up nutrients from soil is highly influenced by their surface, i.e. the root density in soil (Tian et al., 2006). Though our data did not show significant alterations in root density with increasing plant diversity (data not shown), above ground biomass increased with increasing species richness leading to an increase in shoot : root ratios. This effect has already been described by Bessler et al. (2009) where it was suggested that plants growing in diverse communities show higher shoot growth due to interspecific competition for light. This would result in a lower N-uptake per gram plant biomass, assuming that root activity is constant, leading to a higher dilution of N by means of higher above ground biomass amounts. In contrast to this it has been shown that the shoot : root ratio can also increase under higher soil nutrient contents (Brouwer, 1983; Wilson, 1988; Callaway et al., 2003; Kahmen et al., 2005), leaving the dilution effect as the sole possibility to explain a decreasing N-concentration of plant biomass. Indeed we found a slight but significant increase in mineral soil N concentrations (Table 5.2). Thus the latter mechanism might be important in our study, though our data only reflect a small time window in the growing season and mineral N amounts are known to have a high temporal variability (Schimel and Bennett, 2004). In any case the general question on mechanisms driving the decrease of N concentration in plant biomass with increasing diversity needs to be based on the absolute N-uptake of plants and microbes and their correlation to plant and microbial basic characteristics.

The size of the microbial community increased with increasing plant diversity, (Table 5.2). The GLM fitted to these data shows that only species richness and combination of functional groups could explain a significant part of the variation. It has long been known that the high abundance of microbes in rhizosphere (19 - 32 times higher than in root free soil; Bodelier et al., 1997) is most likely due to the effect of high amounts of root exudates serving as N and C source for microbial growth (Rovira, 1965 and citations therein). Root exudation has shown to be enhanced with increasing nitrogen competition between plants (Lemaire and Millard, 1999; Raynaud et al., 2008) as this stimulates microbial activity resulting in increased N-availability in soil in the long term. As increased plant diversity accompanied by increased plant productivity results in intense N competition (Hooper et al., 2005), this might explain the positive effect of plant species richness on microbial abundance.

Kemmitt et al. (2008) showed that bacterial dominated microbial communities which are characterized by a low C : N ratio of 4 – 8 (Harrison and Bardgett, 2004) tend to fix a higher amount of N derived from organic N sources in their biomass than do fungi dominated communities with a C : N of 8 – 20 (Kemmitt et al., 2008). As the microbial biomass in our investigations had a C : N of 7.0 – 7.4 it has to be expected according to these authors, that 50 – 70 % of the organic N metabolized by microbes is fixed in the microbial biomass and is therefore not plant available. An increasing microbial abundance accompanied by a higher plant biomass implies a higher N-demand of both, microbes and plants and therefore an intensified competition for N with increasing plant diversity. An increasing microbial N demand with increasing plant diversity in combination with the high N fixing potential of the present microbial community tends to lead to an overall increasing microbial N uptake. As a result a reduced N supply of plants via the microbial mineralization of organic N with increasing plant diversity has to expected, at least in the short term (Hodge et al., 2000a).

5.2 Microbial and plant tracer uptake

To investigate soil nitrogen competition between plants and microbes and its changes with increasing plant diversity, we applied N in the form of amino acids which is the most abundant organic N form in soil (Streeter et al., 2000; Bol et al., 2002; Jones et al., 2005a). This approach considers plant and microbial uptake of intact amino acid N as well as N derived from ammonification and mineralization of amino acid tracer and therefore reflects a competitive status as it has to be assumed for natural systems. Our data indicate that independent of plant diversity, the microbial community is supe-

rior in the acquisition of tracer derived N by a factor of 15 to 30 (Fig. 5.2). This was also true for the microbial uptake of tracer C, which was highly correlated to tracer N uptake ($R^2 = 0.83$, $p < 0.01$). According to Näsholm et al. (1998) this would indicate that at least a part of the tracer derived N has been taken up in an intact form by the microbial community. Based on the applied tracer amounts, microbes took up an average of 54 % of the applied tracer N while plants took up only 2.68 %. Overall the microbial superiority in short term N-acquisition is in broad concordance with previous findings (Jones, 1999; Hodge et al., 2000a; Hodge et al., 2000b; Bardgett et al., 2003; Cole et al., 2004; Jones et al., 2005b). Apart from this well documented fact, the interesting result here is that while total tracer-N uptake rates by plants decreased with increasing diversity, microbes showed an idiosyncratic response, but on a much higher level of tracer-N uptake. The decreasing tracer-N uptake of plants was mainly controlled by the negative impact of increasing microbial abundance with increasing species richness and microbial tracer-N uptake (Table 5.3), thus indicating a competition controlled plant N-uptake. However, microbial tracer N-uptake was only effected by block and plant functional groups (Table 5.3) which might be caused by the fact that different functional groups can alter the concentration of soil amino acids (Hertenberg et al. 2002). This, in return, would influence microbial uptake due to different dilution of the tracer, possibly superimposing any biodiversity effect on tracer uptake of microbes.

5.3 Total N and amino acid uptake by plants

A number of studies have shown the utility of N-tracers and the calculation of tracer uptake to investigate the influence of biotic and abiotic factors on the competition for nitrogen between microbes and plants (Owen and Jones, 2001; Bardgett et al., 2002; Bardgett et al., 2003; Cole et al., 2004; Dunn et al., 2006; Harrison et al., 2007; Sauheitl et al., 2009a). In contrast to this, any interpretations of these data with respect to questions on the plants total N nutrition or the contribution of total organic-N uptake to the plants N nutrition based on these data are difficult due to the unconsidered effect of tracer dilution in soil. As amino acid tracer and tracer derived mineral N are diluted by different soil pools (soil amino acids, soil N_{min}), differing in size and isotopic signature, the resulting isotopic signature of these sources for plant uptake differ and so do the measured isotopic enrichments in plant material. As a result, tracer uptake values do not represent the importance of amino acid uptake for the plants' N-nutrition and are not valid for comparisons between plots differing in this dilution effect. We therefore cor-

rected for this effect and calculated the total plant uptake of N and amino acids (tracer derived + soil derived).

Nitrogen is known to be one of the main limiting factors for plant growth (Chapin et al., 1986; Vitousek and Howarth, 1991) and therefore any increase in its plant availability ought to stimulate plant biomass production, as long as no other nutrient becomes limiting. We found higher productivity in aboveground plant biomass with increasing plant diversity, but at the same time a decreasing total N-uptake of plants (Fig. 5.3). It was hypothesised that the N-uptake rate of plants is controlled by the overall N-status of the plants (Glass et al., 2002; Persson and Näsholm, 2002), i.e. nitrogen uptake is enhanced under conditions of a low N-nutrition status. Thus, it should be expected that the N uptake of plants is either influenced by the size of sink organs like aboveground biomass (Tian et al., 2006), the root density in soil or the N concentration of the plants' biomass. However, mixed effect model results revealed that these plant variables could only explain a minor part (1 %) of the observed variations, while the largest part was explained by microbial abundance and microbial tracer C- and N-uptake (25 %). At this, a higher tracer C uptake by microbes had a positive effect on the plants' total N uptake (Table 5.4A) which is due to the fact that a certain percentage of the amino acid N is known to be released as N_{min} into the soil during microbial tracer metabolisation (Kemmitt et al., 2008). As a consequence, a higher absolute microbial tracer turnover will also lead to a higher absolute N_{min} release and therefore a higher N availability for plants. Overall this positive effect was overcompensated by the negative effect of a microbial community growing in size and N demand with increasing plant diversity (Table 5.4A). Our data therefore suggests that microbes are superior competitors for N uptake and at the same time control the plants' total N uptake. The negative plant diversity effect on N uptake of plants has to be seen as an effect of the increasing N demand of a growing microbial community.

In contrast to the decreasing total N uptake values, the relative importance of amino acids increased with increasing plant diversity. A number of studies have shown that microbes are more competitive in amino acid uptake than plants (Bardgett et al., 2003; Henry and Jefferies, 2003; Dunn et al., 2006; Harrison et al., 2008). Our results support these findings and suggest that microbes are the dominant drivers of variations in the plants' amino acid uptake. However, microbial abundance and tracer uptake do not sufficiently explain the increasing plant amino acid uptake with increasing diversity (Table 5.4B). Instead, 21 % of the variation was explained by rooting density, above-

ground biomass and most importantly, N-concentration in plant tissue. While an increased rooting density had a positive effect on amino acid uptake, plant N-concentration had a negative effect (Table 5.3). This suggests that the root surface and therefore the potential number of amino acid transporters per se, but also plant internal regulation of amino acid uptake play an important role and have a higher influence on variations of plant amino acid uptake than microbial competition. It has already been suggested that a high N nutrition status or mineral N uptake of plants leads to a down-regulation of the activity of amino acid transporters in plant roots (Persson and Näsholm, 2002; Persson and Näsholm, 2003; Liu and Bush, 2006; Sauheitl et al., 2009a). Although our data indicate that plants are capable of regulating their amino acid uptake irrespective of the microbial amino acid turnover, this ability seems to be limited to a certain extent and plants are therefore not able to reverse the status of competition for organic nitrogen. At this point it is speculative which factors limit the competitive power of plants for amino acids. A straightforward explanation would be the fact that microbes have a high abundance in the rhizosphere (Bodelier et al., 1997) and that any organic N molecule has to pass this zone of high microbial activity (Kuzyakov, 2002) before the plant root even has the possibility to take it up. This spatial zonation might give microbes a decisive advantage in amino acid uptake, leaving only a limited range of amino acid uptake regulation to plants. However, our results show that within these limits the plants are able to effectively control their amino acid uptake irrespective of microbial competition and that they can enhance their amino acid N uptake with increasing diversity.

Together with the decreasing total N uptake, the increasing total uptake of intact amino acids leads to a growing contribution of amino acid N to the plants N nutrition (Fig. 5.3). Thus the increased amino acid uptake has to be seen as a N partitioning strategy to compensate decreasing mineral N uptake due to increased microbial competition for N. In this, plants bypass the microbial degradation of amino acids and lower the retention potential of the microbial biomass for amino acid N. This indicates that under higher competition for N, plants facilitate the use of alternative N sources through niche partitioning (Hooper et al., 2005 and citations therein) which was recently supported in an experimental study by von Felten et al. (2009). This might explain how plants can successfully handle increasing microbial and interspecific plant competition for nitrogen under higher plant diversity. Obviously this is only possible within a certain range (see

previous paragraph) and as a result the uptake of amino acid-N can not fully compensate the decreasing mineral N uptake with increasing diversity.

5.4 A temporal point of view

It has to be mentioned that our findings on uptake rates are limited to the short term (24 h) acquisition of nitrogen and amino acids by microbes and plants. It has been widely discussed whether such short term results indicate an overall superiority of microbes in N uptake or if plants might win in the long term due to their lower biomass turnover rates (Hodge et al., 2000a and citations therein). Several long term experiments indicate that micro organisms can assimilate large amounts of nitrogen very quickly (within hours) but loose this nitrogen to plants in the long term (weeks to months) due to their high turnover rates (Kaye and Hart, 1997 and citations therein). As a consequence, plants ought to have a better N supply in systems with a high microbial turnover. PLFA fingerprints collected in the experimental plots of the current investigation (Sauheitl et al., in preparation) indicate a higher abundance of protozoa in plots of higher diversity. Badalucco et al. (1996) detected a higher turnover of the prokaryotic biomass and a higher release of microbial derived N into the soil in the case of enhanced grazing by protozoa. Thus we should expect an increasing N availability for plants and therefore increased storage of N in plant biomass with increasing plant diversity. Indeed our data show a trend of increasing N storage in plant biomass per ring from 1 over 2 to higher species mixtures (Table 5.2). This shows that plants in our experiment might overcome their inferior competitive power in N uptake in the long run and benefit from an increasing microbial turnover rate and an increased nitrogen source partitioning with increasing plant diversity. These considerations are supported by results of Roscher et al. (2008) who found an increased depletion of plant available soil N-pools with increasing plant diversity, accompanied by a higher plant productivity over several years. The authors ascribed this increased nitrogen use efficiency to plant complementarity effects including temporal, spatial and chemical niching with respect to nitrogen uptake. This assumption is now confirmed by the results of our investigations.

6 Conclusions

The use of dual labelled amino acids enabled us to investigate the influence of altered plant diversity on the N and amino acid uptake characteristics of plants and microbes and to evaluate the importance of amino acid uptake for the plants N nutrition. We came to the following conclusions:

- Microbes are by a factor of 15 – 30 more effective in the short term acquisition of nitrogen derived from amino acids.
- Despite a positive diversity-productivity correlation for plants, this was not a sign of a better N supply as in fact plants showed a decreasing total N uptake with increasing plant species richness due to intense microbial competition for N.
- Despite the overall higher competitive power of microbes in the uptake and metabolisation of amino acid tracer, plants increased the uptake of amino acids with increasing plant diversity and decreasing total plant N and N_{min} uptake.
- Our data indicate that this is due to plant internal regulation mechanisms rather than due to a changing competitive power of microbes. Thus plants dampened the effects of an increasing competition for nitrogen with an increased N partitioning, thereby enlarging the contribution of organic N to their N nutrition.
- Regardless of the inferior competitive power of plants in the short term uptake of N, the increasing size of the plant N pool with increasing plant diversity indicates that plants benefit from increasing turnover rates of the microbial biomass in the long term.

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7 Supplementary

Table 5.1$_{supp}$: Mixed effect model for microbial soil C content using, above ground biomass, *soil density*, *pH*, species richness (*SR*), the combination of functional plant groups (*fgc*), the block number (*BLOCK*) and combination of *BLOCK* and *fgc* as explaining variables.

Source	% Var. explained	Reg. Coeffizient	df	F	Sig.
Intercept	0.08	34.7	1	0.06	0.823
shoot biomass	0.43	-0.1	1	0.33	0.599
soil density	1.60	-48.8	1	1.21	0.334
pH	0.89	10.4	1	0.67	0.459
SR	10.07	2.0	1	7.61	0.050
fct. group comb. (fgc)	14.48		6	0.73	0.634
BLOCK	32.30		3	4.27	0.025
fgc * BLOCK	34.87		10	2.64	0.182
Total explained	94.71				
Total error	5.29				

$R^2 = 0.95$

Table 5.2$_{supp}$: Mixed effect model on *total microbial tracer C uptake* using microbial C, above ground biomass, *pH*, species richness (*SR*), the combination of functional plant groups (*fgc*), the block number (*BLOCK*) and combination of *BLOCK* and *fgc* as explaining variables.

Source	% Var. explained	Reg. Coeffizient	df	F	Sig.
Intercept	4.30	2596.17	1	31.77	0.005
microbial C	2.58	9.37	1	19.28	0.012
shoot biomass	7.29	-4.89	1	54.39	0.002
pH	4.01	-361.32	1	29.90	0.005
SR	2.18	-20.45	1	16.28	0.016
fct. group comb. (fgc)	19.08		6	0.74	0.631
BLOCK	11.52		3	1.79	0.208
fgc * BLOCK	48.50		10	36.19	0.002
Total explained	99.46				
Total error	0.54				

$R^2 = 0.99$

Study 6: Bottom up effects of plant diversity on structure and activity of microbial communities in soil

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Abstract

Microbes are one of the main drivers of nitrogen and carbon cycling in soil and there is increasing awareness in ecological research that plant diversity might have a more specific influence on microbial community structure and functioning as originally supposed.

We therefore investigated changes of the microbial community structure in a grassland diversity gradient from 1 to 16 species using PLFA analysis. In addition, deaminase activity and the metabolic activity of single microbial groups with respect to incorporation of amino acid tracer (^{13}C) was measured.

We found a positive effect of plant diversity on microbial diversity, where the abundance of litter and soil organic matter decomposing gram positive bacteria as well as protozoan abundance increased with increasing plant diversity. Overall the microbial community was dominated by bacteria (54.7 %). Amino acid incorporation was highest for bacteria (70.3 % of the overall microbial uptake) and increased with increasing diversity as did the deaminase activity.

Our results are strong evidence for a bottom up control of microbial diversity by plant diversity leading to increased functional redundancy of microbes and thus to a higher functional system stability with increasing diversity. At the same time, microbial turnover was stimulated by increasing abundance and grazing of protozoa which in combination with an increased deaminase activity ought to increase the loss of microbial N to plants. We therefore conclude that plant diversity can increase N availability in soil by changing composition and turnover of the microbial biomass which in return might explain the observed positive effect of plant diversity on plant productivity.

Keywords: PLFA, microbes, diversity, amino acids, N, protozoa, productivity, stable isotopes

1 Introduction

Plant diversity research focused on two major questions concerning the alteration of plant diversity during the last two decades: 1) What is the reason for the frequently found positive plant productivity effect of plant diversity and how do plants manage to be more productive? And 2) What are the effects of plant diversity changes on typical ecosystem services like C-storage, nutrient retention and system stability?

For both questions diversity and activity of soil microbes might play a central role (van der Heijden et al., 2008) due to several reasons: It is well documented that microbes highly affect the C-cycle in general (Högberg et al., 2001) and that most of the litter carbon from plants is transformed to soil carbon by the action of microbes (Hättenschwiler, 2005). Therefore, Gleixner et al. (2005) suggested, that diversity of both, plants and microbes influence soil C-dynamics and thus C-storage in soil. With respect to the influence on turnover times of soil pools these authors even assume a dominant microbial effect. At the same time microbes are main drivers of the soil N-cycle (Tiedje, 1988; Kowalchuk and Stephen, 2001) and in this the microbial biomass can act as a short term or seasonal N-storage pool (Zogg et al., 2000; Bardgett et al., 2005), therefore increasing the system's nutrient retention capacity. The latter one is also enhanced due to the fact that microbes have been shown to positively affect plant productivity in biodiversity experiments (van der Heijden et al., 2008; and citations therein) resulting in higher nutrient storage of the plant biomass. The positive effects on plant productivity mainly concern nutrient acquisition and can be direct, like N-fixing by symbiotic bacteria (Sprent, 2001), improved nutrient acquisition via mycorrhizal fungi (Vogelsang et al., 2006) or rather indirect like N-fixing via free bacteria (Cleveland et al., 1999) or release of plant available N forms from N containing organic polymers (Schimel and Bennett, 2004). As most of these benefits are mediated by different microbial groups, the possibility of plants for niching and nutrient partitioning, which is an important mechanism to explain increasing plant productivity (Hooper et al., 2005), highly depends on the diversity of the microbial soil community and its activity. Thus, in experiments where plant diversity is increased artificially and an increasing plant productivity is found, a bottom up regulation of the microbial diversity has to be assumed to be consistent with the aforesaid effects of microbes. At the same time rhizosphere living bacteria are also potential competitors for nutrients (Kuikman et al., 1990; Liljeroth et al., 1990; Breland and Bakken, 1991) and thus might also limit plant productivity. Due to the high availability of easy to degrade carbon originated from root

exudation, the number of microbes in the rhizosphere is 19 – 32 times higher than in bulk soil (Bodelier et al., 1997). Despite the surplus of C-sources in the rhizosphere, microbes are suggested to be N-limited (Kuzyakov, 2002) as root exudates usually have a higher C : N ratio than the bacterial biomass (Fenchel, 1982; Kemmitt et al., 2008). Therefore an intense competition for nitrogen as the main limiting nutrient for plant growth (Vitousek and Howarth, 1991) has to be expected.

Thus microbes affect plant productivity in both ways, first by their community structure and diversity which enables niching and nutrient partitioning of plants and second by their abundance and therefore N-demand and activity with respect to metabolisation of N-containing soil substances.

We therefore investigated the effect of increasing plant diversity on the structure and diversity of the microbial community in a grassland ecosystem, increasing plant species numbers from 1 to 2, 4, 8 and 16 species. At the same time we measured the microbial metabolic activity with respect to deaminase activity and degradation and incorporation of amino acids as these are an available N-source for microbes and plants (Lipson and Näsholm, 2001) and are the main precursor substance for the formation of mineral soil N.

2 Material and methods

2.1 Study area

Info on the study area, the configuration of the investigated plots and the labelling and sampling procedure are given in Sauheitl et al. (in preparation) but will be repeated here for completeness.

The experiment was carried out in August 2007 on plots of the *Jena Experiment* (Roscher et al., 2004), which is located on a flood-plain of the Saale river near Jena, Germany (11°34'60" East; 50°55'60" North; altitude 130 m a.s.l.). The soil of the experimental site was classified as Eutric Fluvisol (FAO-UNESCO, 1997) and was highly fertilized during the last 40 years of agricultural utilisation until 2002 when the *Jena Experiment* started. Labelling was done on plots of 5 x 5 m size, representing a biodiversity gradient of 1 – 16 plant species. Biodiversity was altered in 5 steps from 1 to 2, 4, 8 and finally 16 species using plant species that represented grasses, small herbs and tall herbs as functional groups in random mixtures (Roscher et al., 2004). Each diversity level was present in six fold replication, except for the 16 species mixture with four rep-

licates. One year before the experiment started, three 6 week old phytometer plants, namely *Plantago lanceolata, Geranium pratense* and *Festuca pratense*, were planted in three fold replicate in a quarter of each of the plots (Figure 6.1). This was done as it had been shown in literature that the uptake potential for amino acids of different plant species widely varies (Weigelt et al., 2003; Weigelt et al., 2005). Changes in the amino acid uptake between plots of different biodiversity, which were object to another investigation (Sauheitl et al., in preparation), might therefore be rather due to changes in sampled species than due to the effect of altered biodiversity. The use of plant species that are present in all plots (phytometers) removes this effect, leaving plant diversity as the sole manipulated variable.

Four weeks before labelling started each plot was equipped with polyethylene tubes (PE) of 30 cm diameter that allowed to measure gas efflux from the soil and impeded a lateral dispersal of the applied tracer. The tubes were installed in the soil to a depth of 30 cm, each enclosing one phytometer cluster in its centre. The tubes had an open top that could be closed gas tight when gas measurements were started.
One week before tracer application, the soil cover of the plots by plants was estimated visually.

2.2 Labelling

One of the three phytometer clusters of each plot was used for measurements of natural isotopic composition of plant and soil material, while the other two received a solution of dual labelled (^{13}C and ^{15}N) amino acids. The used amino acid solutions contained equal amounts of glycine, valine and phenylalanine either in a labelled form (98 at% ^{13}C and ^{15}N) or in an unlabelled form for application in the background rings. The respective solutions were injected into the soil to a depth of 5 cm using a dispenser (Eppendorf multipette® 4780, Eppendorf, Hamburg, Germany) that was connected via a silicon tubular to a stainless steel needle with a side hole. Funnels were placed round the injection needle to impede contamination of above ground biomass. To avoid clogging of the syringe holes were drilled into the soil using a small screwdriver before tracer application. A number of 17 injections of 2 mL ensured the homogenous dispersion of the solution within the PE tubes resulting in a total volume of 34 mL containing app. 130 µmol of each amino acid. The two labelled rings differed in sampling time, i.e. while one ring was sampled on five consecutive time steps within four weeks after application, the other one was only sampled once after 24 hours. As it has been shown that intact amino acid uptake can best be measured within a short time after tracer applica-

tion (Streeter et al., 2000) the measurements of amino acid uptake together with PLFA analysis were only done in these short term (ST) ring. The long term (LT) rings were used to investigate the fate of tracer derived ^{13}C and ^{15}N in soil and soil gas efflux over a longer span. However, we will only present the results of the ST experiment here while the data on the long term turnover of amino acid tracer are published elsewhere (Barnard et al., in preparation).

2.3 Sampling

Sampling started with the cutting of the aboveground plant biomass. After biomass sampling, four soil samples were taken to a depth of 30 cm using a metal corer. One soil sample was taken below one of the three phytometers, respectively, and the fourth sample was located in the centre between the phytometers. Soil and plant samples were immediately cooled in a cooling box and brought to the lab within two hours. Plant samples thereafter were frozen in liquid N_2 and stored at -30 °C until further analysis, while soil samples were sieved to < 2 mm at which roots were collected and washed according to Sauheitl et al. (2009b). After washing, roots were frozen in liquid N_2 and stored at -40 °C until further analysis. Residual soil was frozen in liquid N_2 and stored at -40 °C until further analysis.

2.3 Deaminase activity

Activity of deaminase in soil was measured according to Kilham and Rashid (1986). In brief a model substrate (1,2 diaminonitrobenzol) was added to 1 g of soil and the mixture was suspended in a phosphate buffer, which was adjusted to the pH of the respective soil sample. Thereafter the mixture was incubated for 20 h at 20 °C during constant shaking. After this time remaining substrate was extracted from soil using methanol and the amount of not deaminated substrate was measured on a photometer at a wavelength of 405 nm. Deaminase activity was calculated as the portion of deaminased substrate in %.

2.4 Phospho Lipid Fatty Acid (PLFA) analysis

2.4.1 Extraction, purification and derivatization of phospholipids

Extraction and purification of phospholipids followed the procedure as outlined in Frostegard et al. (1991). After purification, phospholipids were saponified to free fatty acids and transformed to fatty acid methyl esters following the derivatization procedure of Knapp (1979). Recovery was calculated using 25 µg of 1,2-dinonadecanoyl-

L-α-phosphatidylcholin (Larodan, Sweden) as first and 25 µg of tridecanoic acid methyl ester (Larodan, Sweden) as second internal standard. Parallel to the samples, mixtures of 21 fatty acids (Table 6.1_{supp} in supplementary) were derivatized containing 1, 2, 5, 10, 15 and 20 µg of the respective fatty acids.

2.4.2 PLFA Measurements

Amount and $\delta^{13}C$ signature of the single fatty acids were measured on an isotopic mass spectrometer (IRMS; Delta Plus^{TM}, Thermo Finnigan, Dreieich, Germany) coupled to a gas chromatograph (GC; Trace GC 2000, Thermo Finnigan) via a combustion interface. Detailed information on the instrumental setup and the used GC-column can be found in Sauheitl et al. (2005) and Birk et al. (2009), respectively. Online referencing of $\delta^{13}C$ values was done by the injection of several reference gas pulses directly into the IRMS during measurement as described in Glaser et al. (2002) and Sauheitl et al. (2009a).

2.5 Calculations

PLFA content in soil was calculated as µg PLFA per g dry soil and the single PLFA contents were converted to their portion in the total PLFA content of the respective soil sample, further on denoted as *relative content* of a PLFA. If several single PLFAs were characteristic for one microbial group, PLFA contents were summed to reflect the abundance of the specific group.

The Simpson Index D (1) and Simpson's Evenness (2) were calculated to depict changes of microbial diversity (Nentwig et al., 2004):

$$D = 1 - \sum_{i=1}^{i=N} (p_i)^2 \qquad (1)$$

$$\text{Evenness} = \frac{D}{\left(1 - \frac{1}{N}\right)} \qquad (2)$$

Where p is the relative abundance of a PLFA i in soil and N is the number of PLFA present in a sample.

The Bray-Curtis-Index ($S_{j,k}$) (Carney and Matson, 2006) was calculated as a degree of similarities in the PLFA composition of two plots:

$$S_{j,k} = 1 - \frac{\sum_{i=1}^{p} \left| y_{i,j} - y_{i,k} \right|}{\sum_{i=1}^{p} \left| y_{i,j} + y_{i,k} \right|} \quad (3)$$

Where $y_{i,j}$ or $y_{i,k}$ is the mole percentage of a PLFA *i* in a site *j* and *k,* respectively.

All isotope measurement were done using the δ-notation, δ-values were transformed to at% values according to Craig (1953) and used for all further calculations. Measured δ-values of PLFAs were corrected for the influence of derivative C in analogy to Glaser et al. (2002) and ^{13}C enrichment in single PLFAs was calculated as the difference between labelled and unlabelled soil samples. The amount of tracer-C uptake into PLFA was calculated based on the ^{13}C enrichment of PLFAs, following a two pool dilution model (Gearing, 1991). Tracer uptake is either depicted as the proportion of tracer C incorporated into an individual PLFA, based on the total C amount of this PLFA (*tracer uptake efficiency*) or depicted as the contribution of tracer C incorporated in a single PLFA to the total tracer C amount incorporated into the total PLFA pool (*contribution to total tracer uptake*). While the first of these variables shows how effective a microbial group takes up tracer ^{13}C, irrespective of its abundance, the second one shows the ecological importance of single groups with respect to tracer uptake. If single PLFAs were characteristic for one microbial group (see below) the respective uptake rates were calculated as sum.

2.6 Statistics

All statistical analysis were done using *Statistika* (Vers. 6.0, Statsoft GmbH, Hamburg). Measured variables were screened for outliners using the Nalimov test (Gottwald, 2000) and data were tested to be normally distributed and variances to be homogenous using the Shapiro-Wilk and the Levene test, respectively. Grouping of PLFAs to define microbial groups with similar eco-physiological properties was done using principal component analysis (PCA). The effect of plant species richness on deaminase activity, PLFA composition and tracer incorporation into PLFA was tested by means of an analysis of variance (ANOVA) in which the spatial position of the analysed plot within the field site (block) was implemented as random effect. Significance and effect size of not manipulated system variables (pH, soil density, shoot biomass, etc.) on deaminase activity, PLFA composition and tracer incorporation into PLFA were calculated in a mixed effect model using block as a random variable. Similarities in the PLFA composition between single plots were analysed using a hierarchical cluster

analysis and an analysis of similarities (ANOSIM) using the Bray-Curtis-Index as input variable.

3 Results

3.1 PCA of PLFA contents

After a cross search in literature to determine the microbial groups that were represented by the single PLFAs, a PCA was conducted to separate PLFAs representing the same microbial group but showing a different ecological pattern with respect to our treatments. According to literature the following fatty acids were grouped to represent single microbial groups: Bacterial groups were defined by fatty acids i15:0, a15:0, i16:0, i17:0, a17:0 and 10Me18:0 representing Gram positive (G^+) bacteria (Brennan, 1988; Haack et al., 1994) and 18:1 ω9c, cy18:0, 18:1ω7c, 16:1 ω7c and cy17:0 representing Gram negative (G^-) bacteria (Fierer et al., 2003; McMahon et al., 2005). Fatty acids 10Me16:0 and 10Me17:0 represent actinomycetes, while protozoa where indicated by 20:4 ω6 (Fierer et al., 2003), vesicular arbuscular mycorrhiza (VAM) by 16:1 ω5c (Olsson, 1999) and fungi by 18:2 ω6,9 (Federle et al., 1986).

The PCA extracted 5 factors, explaining 78 % of the PLFA variances (Table 6.2_{supp} in supplementary). PLFAs representing G^+ groups were present in factor 1 and factor 2, while G^- bacteria PLFAs were contained in factor 1, 3 and 4. Factor 2 and 5 contained fungi and actinomycetes, respectively, while factor 3 enclosed VAM and protozoa.

3.2 PLFA fingerprint

The total amount of PLFA was dominated by PLFAs representing bacteria (54.7 %) while actinomycete, VAM, fungi and protozoa only accounted for 21.5 % (Table 6.1). The remaining 23.8 % were made up by unspecific PLFAs and were thus not used for the classification of microbial groups. In detail, highest relative amounts were found for the second group of G^- (G^-2: 15.1 %), followed by G^-1 (13.6 %), G^+2 (12.5 %), G^-3 (9.3 %), G^+1 (4.2 %), actinomycete (7.7 %), VAM (7.0 %), fungi (3.9 %) and protozoa (2.9 %) (Table 6.1). An ANOVA revealed a significant influence of plant diversity on the rel. amounts of G^+1, G^+2, G^-1, G^-2, fungi, VAM and protozoa (Table 6.2, Figure 6.1). While G^-1 showed decreasing rel. amounts, G^+1, fungi and protozoa increased in their rel. abundance by app. 2 % with increasing plant diversity, i.e. they

nearly doubled their abundance as found in monocultures. Though the groups G^+2, G^-2 and VAM were affected by plant species richness, there was no consistent trend.

Table 6.1: Contribution of single microbial groups to the whole PLFA amount (rel. amount), the contribution of tracer C to the total PLFA-C amount of a specific group (uptake efficiency) and the portion of tracer uptake of a specific group to the total tracer incorporation of all groups (rel. uptake). SE gives the standard error.

group	rel. amount [%]	SE	uptake eff. [%]	SE	rel. uptake [%]	SE
G^+1	4.17	± 0.38	0.055	± 0.004	2.81	± 0.09
G^+2	12.52	± 0.39	0.174	± 0.012	23.54	± 0.60
G^-1	13.61	± 0.54	0.151	± 0.011	27.61	± 0.55
G^-2	15.10	± 0.38	0.036	± 0.003	7.01	± 0.19
G^-3	9.30	± 0.11	0.088	± 0.007	9.43	± 0.26
fungi	3.85	± 0.30	0.012	± 0.001	0.59	± 0.04
actinomycetes	7.68	± 0.15	0.010	± 0.001	0.89	± 0.03
VAM	7.04	± 0.37	0.010	± 0.001	1.16	± 0.07
protozoa	2.89	± 0.37	0.017	± 0.003	0.95	± 0.20

Table 6.2: ANOVA on the effect of plant species richness (SR) on the rel. amounts of PLFAs, classifying the single microbial groups. Given is the degree of freedom and the F and p-value of the single analysis. Arrows indicated a positive (↑) or negative (↓) effect of species richness.

group	effect	df	F	p	
G^+1	SR	4	5.504	0.004**	↑
	block	3	1.008	0.412	
G^+2	SR	4	7.840	0.001***	
	block	3	0.589	0.630	
G^-1	SR	4	7.843	0.001***	↓
	block	3	3.151	0.049*	
G^-2	SR	4	4.083	0.014*	
	block	3	1.280	0.308	
G^-3	SR	4	1.389	0.275	
	block	3	2.053	0.140	
fungi	SR	4	15.776	0.000***	↑
	block	3	2.006	0.149	
protozoa	SR	4	4.878	0.019*	↑
	block	3	0.756	0.544	
actinomycetes	SR	4	1.139	0.370	
	block	3	1.557	0.234	
VAM	SR	4	5.628	0.004**	
	block	3	0.751	0.535	

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

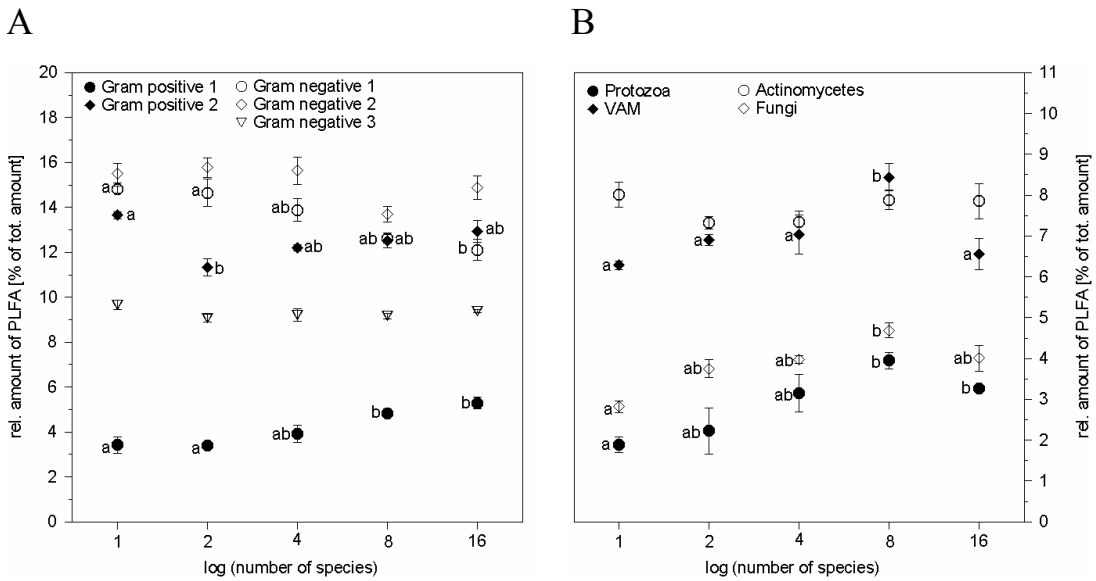

Figure 6.1: Contribution of prokaryotic (A) and eukaryotic (B) microbial groups to the total PLFA content (rel. amount) as affected by the number of plant species. Brackets show standard errors and different lower case letters show significant differences between different plant diversity levels.

3.3 α and β Diversity

The α-Diversity of the microbial community as described by the Simpson Index and the Simpson Eveness increased significantly from 0.913 and 0.961 to app. 0.926 and 0.972, respectively, with increasing plant diversity (Table 6.3A).

For the determination of the β-Diversity, similarities of the PLFA fingerprint between plots of different plant species richness were measured by means of hierarchical cluster analysis and by an ANOSIM of the Bray-Curtis Index of these plots. The hierarchical cluster analysis separated four clusters, of which one contained all plots with 16 species (SR16), the next four of the six SR4 plots, the next four of the six SR1, while the fourth was comprised of plots with medium species richness (Figure 6.1 in supplementary). The ANOSIM revealed that plant species richness had a significant influence on the Bray-Curtis Index and included post-hoc tests showed that plots of SR1 and SR2 differed significantly from SR8 and SR16, while SR4 did not differ from any of these (Table 6.3B).

Table 6.3: ANOVA on the effect of species richness on the microbial Simpson Index (A) and post-hoc test of an ANOSIM of the microbial Bray-Curtis Index (B). Given is the degree of freedom and the F and p-value of the single analysis. Arrows indicated a positive (↑) or negative (↓) effect of species richness.

A)

Variable	Effect	df	F	p	
Simpson Index	SR	4	6.671	0.001**	↑
	Block	3	0.920	0.449	
Simpson's Evenness	SR	4	8.921	0.000***	↑
	Block	3	2.009	0.145	

** p < 0.01

*** p < 0.001

B)

SR	1	2	4	8	16
1	1	0.4235	0.3109	0.0018**	0.0044**
2	0.4235	1	0.5253	0.0029**	0.0051**
4	0.3109	0.5253	1	0.0574	0.1947
8	0.0018**	0.0029**	0.0574	1	0.1859
16	0.0044**	0.0051	0.1947	0.1859	1

** p < 0.01

3.4 Tracer uptake

While all investigated microbial groups incorporated significant amounts of tracer in their characteristic PLFAs, there were significant differences between the single groups with respect to uptake efficiency and rel. tracer uptake. Bacterial groups showed the highest average efficiency for tracer-^{13}C (amino acids) uptake ($G^+2 > G^-1 > G^-3 > G^+1 > G^-2$), followed by fungi, actinomycetes, VAM and protozoa, having an equal efficiency of app. 0.013 % (Table 6.1). Based on the tracer uptake of all groups, the highest portion was taken up by G^-1 (27.6 %), followed by G^+2 (23.5 %), G^-3 (9.4 %), G^-2 (7.0 %), G^+1 (2.8 %) and fungi, actinomycetes, VAM and protozoa, having an equal rel. tracer uptake of app. 1 % (Table 6.1). Thus, 70.3 % of the tracer taken up by the investigated microbial community were taken up by bacteria. The rel. tracer uptake was significantly affected by the number of plant species in the case of G^+1, G^-3, protozoa and VAM (Table 6.3, Figure 6.2). In this, rel. tracer uptake increased for G^+1, G^-3 and protozoa with increasing plant diversity, while it decreased for VAM. Overall prokaryotes took up more tracer with increasing plant diversity. Species richness had a positive effect on the uptake efficiency of protozoa and actinomycetes, while all other groups were not affected (Table 6.4).

Along with the tracer uptake, deaminase activity in soil was measured and we found a significant positive effect of species richness on deaminase activity (Table 6.4 in supplementary).

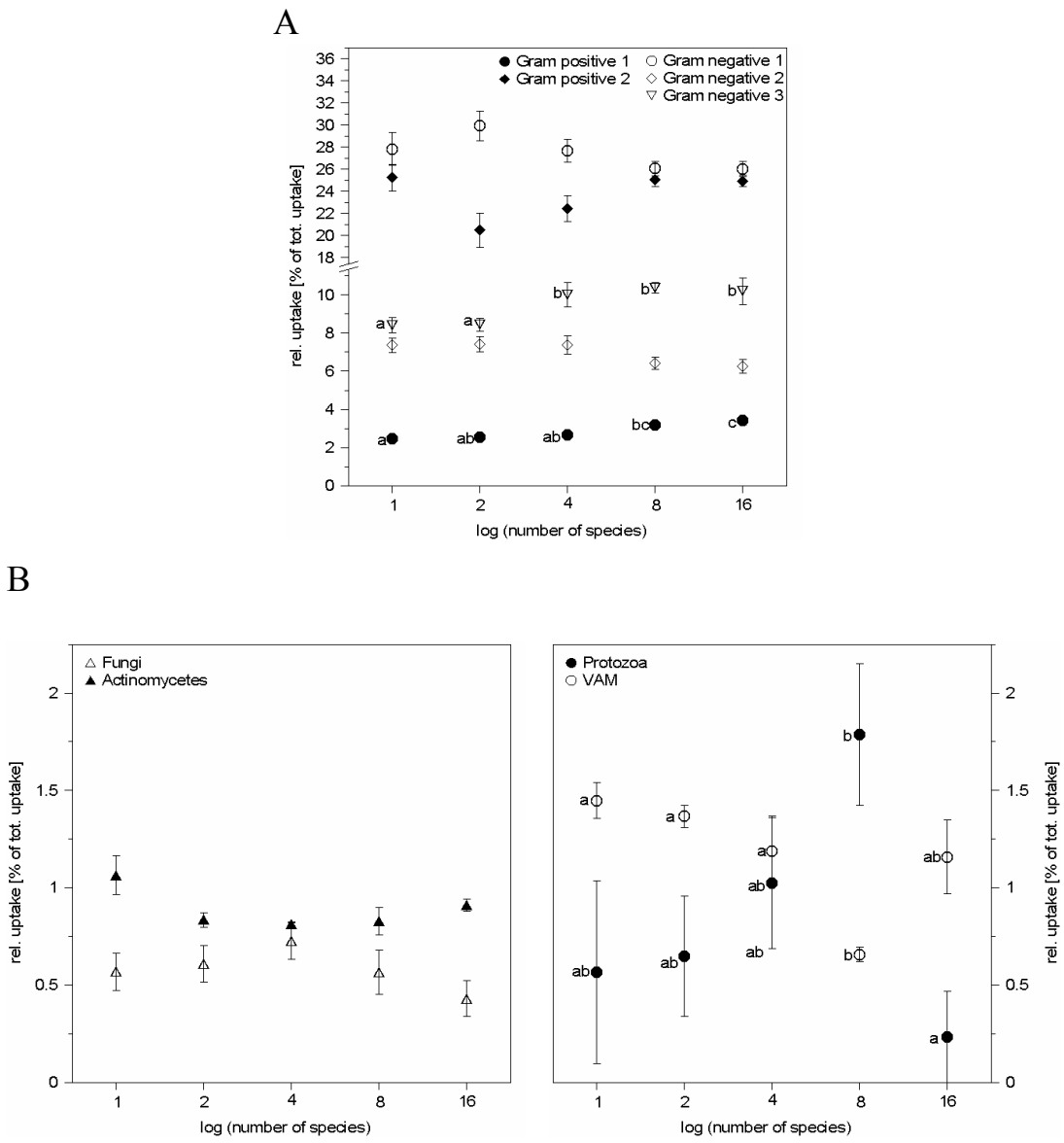

Figure 6.2: Contribution of prokaryotic (A) and eukaryotic (B) microbial groups to the total tracer ^{13}C uptake (rel. uptake) as found in the whole PLFA pool. Brackets show standard errors and different lower case letters show significant differences between different plant diversity levels.

Table 6.4: ANOVA on the effect of plant species richness (SR) on the contribution of tracer C to the total PLFA-C amount of a specific group (uptake efficiency) and the portion of tracer uptake of a specific group to the total tracer incorporation of all groups (rel. uptake). Given is the degree of freedom and the F and p-value of the single analysis. Arrows indicated a positive (↑) or negative (↓) effect of species richness.

			rel. uptake			uptake efficiency		
group	**effect**	**df**	**F**	**p**		**F**	**p**	
G^+1	SR	4	9.065	0.000***	↑	2.286	0.096	
	block	3	1.037	0.397		0.363	0.780	
G^+2	SR	4	5.391	.004**		2.063	0.124	
	block	3	2.466	.092		0.51	0.680	
G^-1	SR	4	1.785	0.171		0.798	0.541	
	block	3	0.182	.907		0.277	0.841	
G^-2	SR	4	1.931	0.144		0.403	0.804	
	block	3	0.797	.510		0.366	0.778	
G^-3	SR	4	9.289	0.000***	↑	0.851	0.510	
	block	3	8.974	.001**		0.288	0.833	
fungi	SR	4	0.826	0.524		0.899	0.483	
	block	3	1.427	.264		0.808	0.504	
protozoa	SR	4	3.79	0.030*	↑	17.009	0.000***	↑
	block	3	1.581	.242		2.753	0.069	
actinomycetes	SR	4	2.617	0.066		3.509	0.025***	↑
	block	3	0.764	.528		0.818	0.499	
VAM	SR	4	6.001	0.002**	↓	0.265	0.897	
	block	3	0.584	0.632		0.588	0.630	

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

4 Discussion

4.1 Microbial community structure and diversity

Classification of microbes by means of PLFA analysis was originally developed from microbial model organisms (Zelles, 1999 and citations therein) that were chosen to differ in physiological characteristics like gram coloring (gram positive and negative bacteria), habit (actinomycetes, protozoa) or association with roots (vesicular arbuscular mycorrhiza). Interpretations of the latter two groups in ecological studies can be done straightforward as the ecophysiological potentials of these groups are well known. However, the classification of prokaryotes (gram positive and negative bacteria) covers a wide range of bacterial groups differing in habitats (aerobic or anaerobic) and substrate use and thus interpretations of the summed PLFAs characteristic for this group with respect to ecological studies is difficult. We therefore improved this classification

by means of a PCA analysis, enabling us to separate gram positive and negative bacteria into new groups that showed a different reaction with respect to our treatments. In addition we were also able to assign PLFA that according to literature can be characteristic for two groups to one specific group by cross comparisons in the PCA with other PLFA that were characteristic for one group or the other.

Our results show that plant species richness had a significant effect on the composition of the microbial community (Table 6.2, Figure 6.1). In this, plant diversity had a positive effect on the abundance of fungi, protozoa and gram positives of group 1 (G^+1) but a negative effect on G^-1. It has been shown that fungi mainly decompose complex organic materials derived from plant debris (Poll et al., 2008). As litter composition widely varies between plant species (Gransee and Wittenmayer, 2000; Eskelinen et al., 2009), increasing plant diversity will result in a higher chemical diversity of litter input into soil. This in return will open new niches for litter decomposing fungi and ought to stimulate their growth (Hooper et al., 2000). This is supported by the results of our investigations as well as by the findings of Zak et al. (2003). Another group that showed a positive effect of plant diversity is G^+1. These bacteria have been shown to be involved in the degradation of soil organic matter (SOM) (Kramer and Gleixner, 2006) and SOM contents are known to increase with increasing plant diversity and productivity (Steinbeiss et al., 2008). Therefore, the positive plant diversity-productivity correlation as found in the Jena experiment (Roscher et al., 2005) ought to increase the abundance of SOM degrading bacteria. Protozoa which also showed a positive response to increasing plant diversity mainly feed on prokaryotes (Kuikman et al., 1990) and the growth of these in return is known to be stimulated by increasing root exudation. Though we have not quantified root exudation, there is strong evidence that plants increase their root exudation under conditions of higher N competition (Lemaire and Millard, 1999; Raynaud et al., 2008) which has been shown to occur in our experiment (Roscher et al., 2008). Indeed our data show a positive effect of the abundance of prokaryotes on protozoa (Table 6.3 in supplementary), which might be the link between plant diversity and protozoan abundance.

All other groups did either not react to plant diversity, showed an inconsistent reaction or decreased in their abundance (G^-1). This is most surprising in the case of gram negative bacteria, as these are known to be adapted to high nutrient concentrations (Griffiths et al., 1999) as present in the rhizosphere, where the concentrations of exudates are high. Thus, this group should benefit from higher root exudation with increas-

ing plant diversity as proposed in this investigation. However, the reaction of prokaryotes might be simply overlaid by the grazing effect of protozoa. This top down control of microbial biomass has already been found by Badalucco et al. (1996) and Vedder et al. (1996). Together with the bottom up control of plant diversity via increasing root exudation (Wardle et al., 2004), this might lead to an inconsistent or flattened reaction of some microbial populations on increased plant diversity.

The reorganization of the microbial community as induced by plant diversity resulted in an increasing microbial α-diversity, therefore indicating a positive bottom up effect of plant diversity on microbial diversity. The change of microbial diversity in our study was caused by changes of the relative abundance of single groups but not by the appearance or disappearance of a whole group. Therefore it could be suggested that a loss of diversity with respect to microbial groups will not result in a loss of ecosystem functions in this case. This is especially true as in soil several organism are expected to perform similar functions (Wardle et al., 2004). However, the microbial groups defined in this investigation are comprised of several species and the decreasing relative abundance of several microbial groups as indicated by decreasing α-diversity might be caused by the disappearance of single species within a microbial group. In this context, De Deyn and Van der Putten (2005) concluded that for species poor systems, functional redundancy is low and effects of species loss are therefore intense. Transferring this to the present study, a loss of plant diversity is therefore likely to be accompanied by an increased risk for the loss of microbial ecosystem functions due to the loss of functional redundancy. In addition to this, the ANOSIM and cluster analysis showed that there are significant and frequent changes in the microbial community composition for different diversity levels, suggesting a high β-diversity. These results support other investigations (Zak et al., 2003; Wardle et al., 2004) and affirm the growing awareness in ecology, that above ground diversity affects below ground diversity in a much more specific way than originally supposed (De Deyn et al., 2003).

4.2 Microbial amino acid metabolisation

The bulk of the bacterial soil community mainly feeds on low molecular substances (LMWOS) (Haller and Stolp, 1985) and the dominance of prokaryotes in tracer ^{13}C uptake as found in our investigation (Table 6.1) verifies this osmotrophic nutrition. Within the bacterial community, tracer uptake is dominated by $G^{+}2$ and $G^{-}1$ which also have the highest uptake efficiencies, i.e. they take up more tracer based on the same abundance than other bacteria. This indicates specialization on the uptake of LMWOSs

which has already been suggested by Griffiths et al. (1999) who found that this Gram negative group reacts more positively on high concentrations of LMWOS than do other groups. Williams et al. (2007) found that G^+2 are especially involved in the initial degradation of fresh clover litter and might therefore be adapted to the high LMWOS concentrations as present in initial litter decomposition. This suggestion is now affirmed by the relative tracer uptake and the uptake efficiencies found in our investigation.

In contrast to prokaryotes, all eukaryotes showed a significantly lower relative tracer uptake. For all of these groups this is partly due to their low abundance compared to prokaryotes (Table 6.1). However, as for actinomycetes and fungi, the low uptake efficiencies also indicate that these groups are not adapted to take up LMWOS but are specialized on the degradation of complex organic molecules (Poll et al., 2008) as already assumed before. It has been discussed, whether mycorrhiza might increase the plants ability to take up amino acids from the soil (Persson and Näsholm, 2001). However, these authors found that the plants amino acid uptake was not affected by their degree of mycorrhization. The low uptake rates and uptake efficiency of VAM as found in our investigation affirm these findings and thus a significant contribution of VAM to the amino acid uptake of plants is unlikely. In contrast, the low uptake rates of protozoa might simply be caused by a time delay of the tracer signal indicating the higher position of protozoa in the food web compared to prokaryotes.

Beside these intraspecific differences, the most important finding in our investigation was the positive effect of increasing plant diversity on microbial tracer uptake. In this, G^+1 and G^-3 showed increasing uptake rates leading to an overall positive response of tracer uptake for prokaryotes. This was accompanied by increasing tracer uptake of protozoa, indicating higher tracer flow rates through the microbial food web and thus suggesting a higher turnover of the microbial population as shown by Kuikman et al. (1990) in laboratory experiments. Protozoan predation was also shown to stimulate deaminase activity in soil (Badalucco et al., 1996) and thus higher microbial turnover ought to boost microbial N loss to plants (Kaye and Hart, 1997 and citations therein). The positive effect of plant diversity on microbial turnover and deaminase activity as found in our investigation is thus likely to increase N availability for plants in the long term (Zak et al., 2003), resulting in a positive feedback of plant diversity on plant productivity.

5. Conclusions

Our results show that plant diversity stimulates microbial diversity. This was most likely due to the bottom up effect of increased root exudation and litter input caused by increasing N competition and plant productivity, respectively. As a result protozoan growth and activity was increased, stimulating microbial turnover as indicated by increasing deaminase activity and amino acid uptake. As increased microbial turnover is accompanied by higher N-mineralization, increasing plant diversity ought to increase N availability for plants via a positive feedback of the microbial mineralization loop, thus stimulating plant productivity.

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6 Supplementary

Table 6.1$_{supp}$: List of fatty acids in the standard mixture, including abbreviations and the microbial groups as specified by the single fatty acids.

fatty acid	abbreviation	represents
tetradecanoic acid	14:0	unspecific
pentadecanoic acid	15:0	unspecific
hexadecanoic acid	16:0	unspecific
heptadecanoic acid	17:0	unspecific
octadecanoic acid	18:0	unspecific
12-methyltetradecanoic acid	a15:0	gram positive
13-methyltetradecanoic acid	i15:0	gram positive
14-methylpentadecanoic acid	i16:0	gram positive
14-methylhexadecanoic acid	a17:0	gram positive
15-methylhexadecanoic acid	i17:0	gram positive
cis-9,10-methylenhexadecanoic acid	cy17:0	gram negative
cis-9,10-methylenoctadecanoic acid	cy19:0	gram negative
10-methylhexadecanoic acid	10Me16:0	actinomycetes
10-methylheptadecanoic acid	10Me17:0	actinomycetes
10-methyloctadecanoic acid	10Me18:0	gram positive
cis-11-hexadecenoic acid	16:1ω5c	VAM
cis-9-hexadecenoic acid	16:1ω7c	gram negative
cis-octadecenoic acid	18:1ω7c	gram negative
cis-9-octadecenoic acid	18:1ω9c	gram negative
cis,cis-9,12-octadecadienoic acid	18:2ω6,9	fungi
cis,cis,cis,cis-5,8,11,14-eicosatetraenoic acid	20:4ω6	protozoa

Table 6.2$_{supp}$: Factor loadings of single PLFAs and the microbial groups they represent as found in a PCA.

fatty acid	group	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5
i15:0	G^+	0.070	**0.877**	0.096	-0.119	0.006
a15:0	G^+	0.148	**0.871**	0.097	-0.054	0.044
i16:0	G^+	-0.090	**0.803**	-0.100	0.225	0.202
16:1ω7c	G^-	0.072	0.015	0.071	**0.863**	-0.111
16:1ω5c	VAM	0.066	-0.088	**0.840**	0.221	-0.157
10Me16:0	actinomycetes	0.176	0.0130	-0.060	0.055	**0.863**
i17:0	G^+	**0.873**	0.249	0.045	0.213	0.030
a17:0	G^+	**0.885**	0.178	0.192	0.093	0.063
cy17:0	G^-	0.424	0.036	-0.027	**0.711**	0.306
10Me17:0	actinomycetes	-0.520	0.282	0.254	-0.085	**0.476**
18:2ω6,9	fungi	0.172	**-0.594**	0.396	-0.431	-0.200
18:1ω9c	G^-	-0.559	-0.117	**-0.575**	-0.201	-0.302
18:1ω7c	G^-	**-0.853**	-0.085	-0.362	0.055	-0.064
10Me18:0	G^+	**0.737**	-0.323	0.165	0.121	0.086
cy19:0	G^-	-0.143	-0.106	**-0.869**	0.157	-0.177
20:4ω6	Protozoa	0.484	-0.046	**0.662**	-0.103	-0.118

Table 6.3$_{supp}$: Mixed effect modell on the abundance of protozoa; df gives degrees of freedom, F is the F-value of the ANOVA and Sig. gives the significance.

Source	% Var. explained	df	F	Sig.	
Intercept	5.19	1	1.769	0.216	
root biomass	0.26	1	0.088	0.773	
aboveground biomass	0.37	1	0.126	0.731	
pH	16.97	1	5.780	0.040*	↑
abundance of prokaryotes	10.39	1	3.539	0.093	
SR	52.14	1	17.758	0.002**	↑
BLOCK	11.74	3	4.000	0.046*	
Total explained	97.06				
Total error	2.94				

$R^2 = 0.89$

Table 6.4$_{supp}$: Deaminase activity depicted as rel. turnover of an added substrate, for different diversity levels.

	SR1	SR2	SR4	SR8	SR16
Deaminase activity [% of substrate turnover]	57.18 ± 4.92	58.11 ± 5.14	71.66 ± 7.86	85.09 ± 2.14	79.22 ± 0.55

Previous own publications:

Sauheitl L., B. Glaser, R. Bol, 2005. Short-term dynamics of slurry-derived plant and microbial sugars in a temperate grassland soil as assessed by compound-specific delta C-13 analyses. Rapid Commun. Mass Sp. 19, 11, 1437-1446.

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Declaration / Erklärung

Herewith I declare that the present work was exclusively prepared by me using no others than the named sources and devices.
In addition I declare that I have not yet tried to hand in this PhD thesis, be it successfully or not. I have not definitely failed to pass an equivalent PhD test at another university.

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.
Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht beständen.

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Leopold Sauheitl

Hiermit erkläre ich, dass die Pflichtexemplare meiner Dissertation inhaltlich mit der Fassung übereinstimmen, für die die Druckgenehmigung erteilt wurde (§ 13 Abs. 3 der Promotionsordnung der Fakultät für Biologie, Chemie und Geowissenschaften der Universität Bayreuth in der Fassung der Bekanntmachung vom 15. Juli 2004).

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