Inter-specific variability in organic nitrogen uptake of three temperate grassland species

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Summary – Zusammenfassung

We tested the inter-specific variability in the ability of three dominant grasses of temperate grasslands to take up organic nitrogen (N) in the form of amino acids in soils of differing fertility. Amino acid uptake was determined by injecting dual labeled glycine-2-^{13}C-^{15}N into the soil, and then measuring the enrichment of both ^{13}C and ^{15}N in plant tissue after 50 hours. We found enrichment of both ^{13}C and ^{15}N in root and shoot material of all species in both soils, providing first evidence for direct uptake of glycine. We show that there was considerable inter-specific variability in amino acid uptake in the low fertility soil. Here, direct uptake of amino acid was greater in the grass *Agrostis capillaris*, which typically dominates low fertility grassland, than *Lolium perenne*, which inhabits more fertile sites. Direct uptake of amino acid for *Holcus lanatus* was intermediate between the above two species. Unlike in the low fertility soil, there was no difference in uptake of either ^{13}C or ^{15}N by grasses in the high fertility soil, where uptake of mineral N is thought to be the major mechanism of N uptake of these grasses. Overall, our findings may contribute to our understanding of differences in competitive interactions between grasses in soils of different fertility status.

Interspezifische Variabilität in der Aufnahme organischen Stickstoffs von drei Grünland-Arten

Die Fähigkeit dreier Gräser, organischen Stickstoff in der Form von Aminosäuren aufzunehmen, wurde in Böden unterschiedlicher Fruchtbarkeit untersucht. Die Aufnahme wurde mittels zweifach markiertem Glycin-2-^{13}C-^{15}N getestet, das in die Böden injiziert und dessen Anreicherung im Pflanzengewebe nach 50 h gemessen wurde. Wir fanden eine Anreicherung von ^{13}C und ^{15}N in Wurzel- und Sprossgewebe aller Arten und auf beiden Böden. Auf dem unfruchtbaren Boden zeigten sich deutliche artspezifische Unterschiede, wobei *Agrostis capillaris*, das typischerweise ungedüngte Wiesen dominiert, eine höhere direkte Aufnahme der Aminosäure aufwies als *Lolium perenne*, eine Art, die vorzugsweise gedüngte Standorte besiedelt. Die Aminosäureaufnahme von *Holcus lanatus* lag zwischen diesen beiden Arten. Im Gegensatz dazu zeigte sich kein artspezifischer Unterschied auf dem fruchtbaren Boden, wo die Aufnahme von mineralischem N als primäre Quelle der Stickstoffversorgung dieser Gräser gilt. Insgesamt können unsere Ergebnisse zu einem besseren Verständnis der Konkurrenzverhältnisse zwischen Gräsern auf Böden unterschiedlicher Nährstoffverfügbarkeit beitragen.

Key words: arbuscular mycorrhiza / glycine / grasses / stable isotopes / ^{15}N / ^{13}C PNSS P03/22P

1 Introduction

Soil organic N is of importance for plant nutrition not only in N limited environments such as the arctic and alpine ecosystems (*Schimel* and *Chapin*, 1996; *Raab* et al., 1999) and boreal forests (*Näsholm* et al., 1998), but also in some agricultural systems (*Näsholm* et al., 2000, 2001; *Bardgett* et al., 2003). The importance of organic N for plant nutrition in agricultural soils has been highlighted in pot experiments with grasses (*Näsholm* et al., 2000) and in field experiments with wheat plants (*Näsholm* et al., 2001) where some 19–23 % of plant N-uptake was estimated to be as intact amino acid. Recent field studies by *Bardgett* et al. (2003) also revealed that grassland plants can take up organic N in the form of amino acids, but that more is captured in this way by plants of low productivity grasslands where amino acids were the dominant soluble N form in soil. By contrast, in fertilized grasslands inorganic N is the dominant plant available pool. Consistent with laboratory experiments on organic N use by agricultural plants (*Hodge* et al., 1998, 1999), the bulk of amino acids that was added to soil was sequestered by the microbial biomass, with its subsequent release over longer time-scales being the major pathway for N acquisition by plants (*Bardgett* et al., 2003).

It is known that grassland species differ in their ability to take up organic and inorganic N forms (*Falkengren-Grerup* et al., 2000; *Näsholm* et al., 2000; *Bol* et al., 2002). Consequently, *Bardgett* et al. (2003) proposed that differences in direct uptake of amino acids by plants of low and high productivity grasslands might result, in part, from inter-specific variability in the ability of dominant grasses to utilize organic N. It was also suggested by these authors that organic N uptake by plants might be greater in the relatively infertile, low productivity grasslands through the prevalence of mycorrhizal associations with grasses in these sites. Arbuscular mycorrhizal infection of plants is known to be substantially greater in unfertilized than agriculturally improved grassland (*Read* and *Haselwandter*, 1981; *Bardgett* et al., 1997; *Donnison* et al.,

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2000) and studies demonstrate these fungi can take up some amino acids directly (*Hawkins* et al., 2000).

Inter-specific variability in the ability of dominant grasses of temperate grasslands to take up organic N in the form of an amino acid was studied in a pot experiment. We tested whether the ability of different grasses for amino acid uptake varied in soils of differing fertility and degrees of mycorrhizal colonization. We hypothesized that species that typically dominate low productivity grassland (i.e., *Holcus lanatus L.* and *Agrostis capillaris L.*) will be more able to take up amino acids than the dominant species of productive, agriculturally improved grasslands (*Lolium perenne L.*). We also tested whether there was any difference in colonization of roots by mycorrhizal fungi between the grasses in the two soils, since variations in colonization of roots by these fungi may be instrumental in explaining inter-specific differences in organic N uptake. Common to many studies (e.g., *Nordin* et al., 2001; *Näsholm* et al., 1998, 2000, 2001), we used glycine to measure amino acid uptake since it is one of the most abundant amino acids found in the soil solution of temperate grasslands (*Streeter* et al., 2000; *Bardgett* et al., 2003), in part because it is returned to the soil through the urine of grazing animals (*Bristow* et al., 1992).

2 Materials and methods

2.1 Site description

Soil was taken from an agriculturally improved and a low productivity, unimproved grassland at Littledale, in north Lancashire, United Kingdom, that was used by *Bardgett* et al. (2003) to study in situ uptake of organic N. The improved grassland, which is fertilized on an annual basis with inorganic N fertilizer, was a *Lolium-Cynosurus* grassland (National Vegetation Classification [NVC] MG6; *Rodwell*, 1992) with *Lolium perenne L.* as the dominant grass species. The unimproved, non-fertilized grassland was a *Festuca-Agrostis-Galium* grassland with a *Holcus-Trifolium* sub community (NVC U4b; *Rodwell*, 1992) where *Agrostis capillaris L.* was the most common species. Moreover, the transitional stages between both improved and unimproved grassland were frequently dominated by *Holcus lanatus L.* (*Rodwell*, 1992). The soil of the improved grassland had an average pH of 5.4 (H_2O 1:2.5 w:v), an organic carbon (C) content of 4.4 % (dry wt.), and a bulk density of 1.15 g cm^{-3}. The soil of the unimproved grassland had an average pH of 4.7, an organic C content of 14.5 % (dry wt.), and a bulk density of 0.755 g cm^{-3}. The unimproved site also had a litter layer of at least 3 cm, which was absent on the improved site. Littledale is located in the upland fringes of north-west Britain with an annual precipitation around 1080 mm and a mean annual maximum daily temperature of 12.1 °C.

2.2 Experimental design

The experiment was set up in a factorial design, combining three grass species (*L. perenne*, *H. lanatus*, *A. capillaris*) grown in two soils (improved and unimproved) with 2 types of glycine (dual labeled glycine-2-^{13}C-^{15}N or the unlabelled glycine control) in four replicates. Plants were grown in soil collected from the surface 10 cm of the unimproved and improved soil described above. Prior to potting, soil was passed through a 6 mm sieve and watered to 50 % field capacity moisture content. Soil microcosms were prepared by weighing soil equivalent to 210 g dry weight into pots (10 cm diameter, 8 cm height) lined with clear plastic. Seeds were surface sterilized with 70 % ethanol for 30 seconds, rinsed 5 times in Milli-Q water, and then germinated at 18 °C for 7 days on filter paper soaked with Milli-Q water. Eight germinated seeds were planted per pot. Pots were placed in a greenhouse with an average 16/8 hour day/night cycle of 18/10 °C. After 14 days plants were thinned to four plants per pot. Pots were watered every day.

2.3 Isotope labeling and harvest

After 40 days, when plants were established, all pots were injected with 5.14 mg of glycine-2-^{13}C-^{15}N or 5.36 mg unlabeled glycine (equivalent to 4.8 μg N (g soil)$^{-1}$). Labeled glycine (from Sigma Chemical Company, Dorset, England) was 99 % enriched in both ^{15}N and ^{13}C. We injected 1 ml of solution into the center of each pot with a syringe (5 cm long, 18 gauge sideport needle) that was slowly withdrawn to ensure uniform spread throughout the soil. Rooting was intense in the pots and fairly uniform for all species and treatments, hence probabilities for uptake of the solution did not noticeably differ between species and soils. After 50 hours incubation, shoots were clipped and roots washed with $CaCl_2$ and water, dried at 80 °C for 48 hours and then analyzed for ^{13}C and ^{15}N. For unlabeled treatments, a sub-sample of roots was taken before washing and drying to determine mycorrhizal colonization. The roots were washed in water, cleared (10 % KOH at 80 °C for 2 hours), acidified (10 % HCl), stained for 2 hours (trypan blue in lactoglycerol), de-stained (50 % glycerol) and scored for mycorrhizal colonization at ×300 magnification (*McGonigle* et al., 1990). Root length was estimated by the line intersect method (*Tennant*, 1975).

Isotope analysis was performed on a continuous flow-isotope ratio mass spectrometer (CF-IRMS), using an automated N/C analysis-mass spectrometry system (ANCA-MS; Europa 20/20, Crewe, UK). Ground wheat flour (with 1.08338 atom% ^{13}C and 0.3674 atom% ^{15}N) and 0.5, 1.0, and 5.0 atom% ammonium-^{15}N nitrate was used as the working standard. Values of atom percentage and concentrations of C and N were used to calculate moles excess of ^{13}C and ^{15}N as described by *Näsholm* et al. (2000). Mean values of ^{15}N and ^{13}C abundances of the unlabeled control plants were used as references for ^{13}C and ^{15}N excess and were calculated separately for shoots and roots of each grass species.

2.4 Statistical analysis

Analyses of variance and post-hoc Tukey (HSD) tests were used to detect significant differences between the three grass species. To test for differences between the soils (improved and unimproved), a t-test for independent variables was applied. If assumptions of normality and homogeneity of variances were not met for dependent variables (for ^{15}N

excess of shoots only), log transformed data were used for the analysis. All statistical analyses was performed with Statistica for Windows (Version 5.5, Statsoft, USA.).

3 Results

3.1 Biomass and mycorrhizal colonization

Above ground biomass of the three grass species revealed some significant differences, depending on the soil used. Both *H. lanatus* and *L. perenne* produced less shoot biomass on the unimproved compared with the improved soil, while *A. capillaris* produced slightly more shoot biomass on the unimproved soil (Tab. 1; Fig. 1B). On the improved soil, *H. lanatus* had a significantly greater above ground biomass than *A. capillaris*, while on unimproved soil there was a significant difference only between *H. lanatus* and *L. perenne*. In contrast, below ground biomass production revealed no signifi-

Table 1: T test for significant differences between soils of highly productive, improved grassland (I) and low-productivity, unimproved grassland (U) for the means over all three species of different dependent variables. The second column gives the direction of change between grassland types.

Tabelle 1: T-Test zur Prüfung signifikanter Unterschiede zwischen Böden produktiver, fruchtbarer Wiesen (I) und unproduktiver, unfruchtbarer Wiesen (U) für die Mittelwerte der Variablen aller drei Arten. Die zweite Spalte gibt die Richtung der Veränderung zwischen den beiden Wiesen an.

	direction	t	df	p
Mycorrhizal colonization	U > I	–3.02	22	0.006
Dry weight / shoot	I > U	2.33	22	0.029
/ root		2.03	22	0.054
^{15}N excess / shoot	I > U	3.88	22	0.001
/ root	I > U	2.21	22	0.038
^{13}C excess / shoot	I > U	2.57	22	0.017
/ root		–0.92	22	0.370

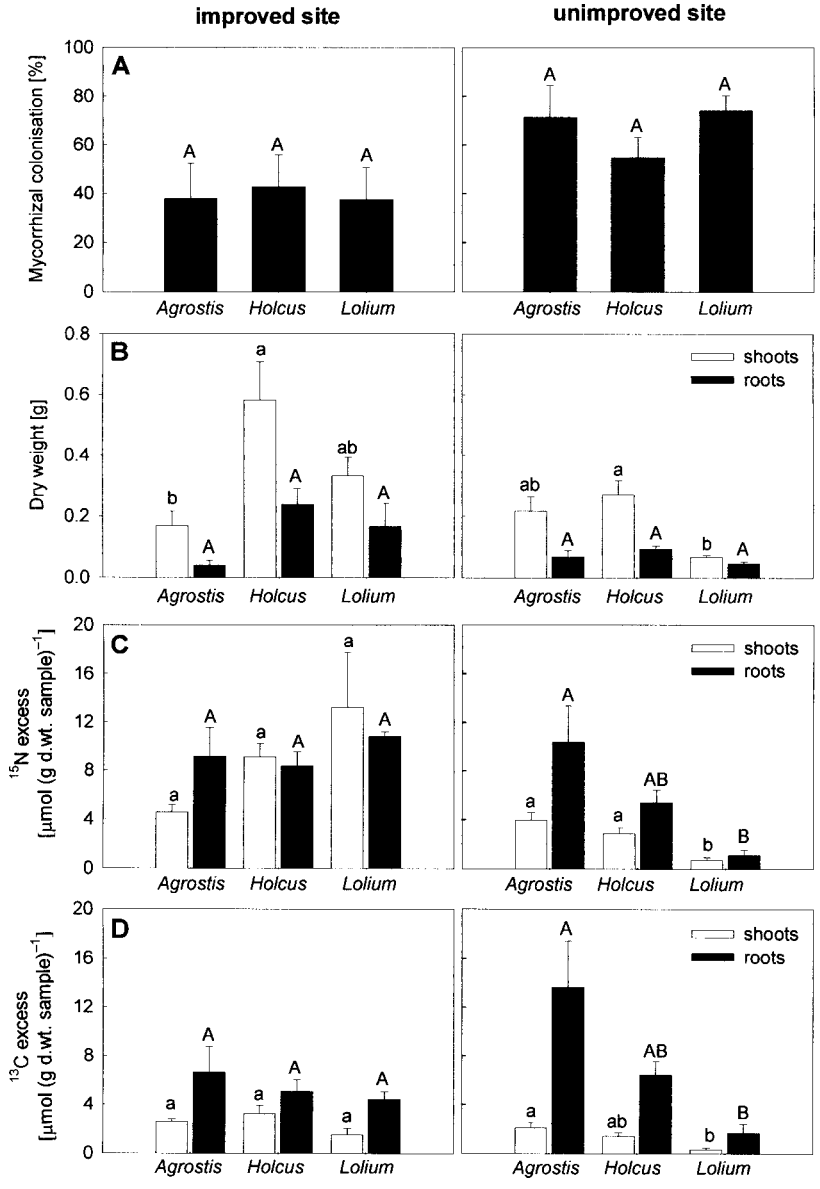

Figure 1: Means (+/- se) of mycorrhizal colonization (A) of all control (unlabeled) plants and of dry weight (B), ^{15}N excess (C) and ^{13}C excess (D) of all labeled plants per species for soils from the improved and unimproved site. Different letters indicate significant differences between species of each given panel (separate comparison of shoots (lower case letters) and roots (upper case letters); ANOVA, post-hoc Tukey (HSD) test, $P < 0.05$, n = 4).

Abbildung 1: Mittelwerte (+/- se) des Mykorrhizierungsgrades aller unmarkierten Kontrollpflanzen (A) und des Trockengewichtes (B), des ^{15}N-Überschusses (C) und des ^{13}C-Überschusses (D) aller markierter Pflanzen pro Art, jeweils für den ungedüngten und gedüngten Boden. Verschiedene Buchstaben symbolisieren signifikante Unterschiede zwischen den Arten (separater Vergleich der Sprosse (kleine Buchstaben) und Wurzeln (große Buchstaben); ANOVA, post-hoc Tukey (HSD) test, $P < 0.05$, n = 4).

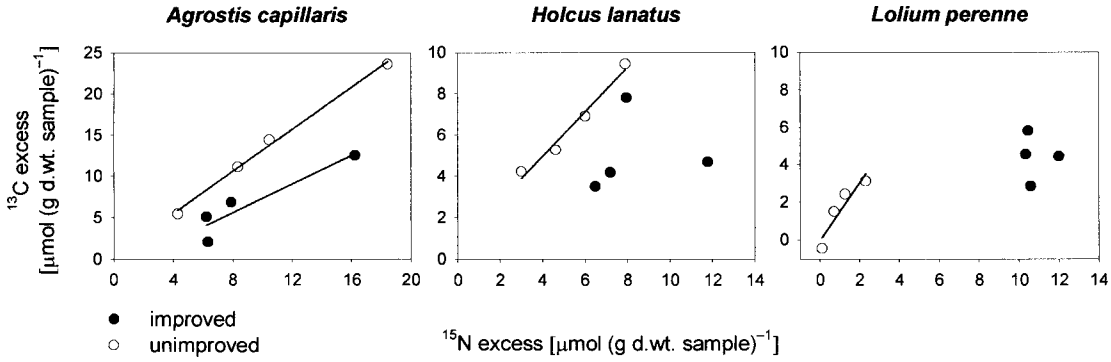

Figure 2: The relationship between excess ^{15}N and excess ^{13}C of root tissue of *A. capillaris*, *H. lanatus*, and *L. perenne* grown on soil of improved (closed circles) or unimproved grassland (open circles). Each symbol represents one pot. The regressions relating to glycine-treated pots are shown: *A. capillaris* unimproved (slope = 1.28, r^2 = 0.99) and improved (slope = 0.86, r^2 = 0.88). *H. lanatus* unimproved (slope = 1.08, r^2 = 0.98). *L. perenne* unimproved (slope = 1.52, r^2 = 0.87).

Abbildung 2: Beziehung zwischen dem Überschuss an ^{15}N und ^{13}C in den Wurzeln der Arten *A. capillaris, H. lanatus und L. perenne*, die jeweils in Böden der fruchtbaren (gefüllte Kreise) oder unfruchtbaren Wiese (offene Kreise) wuchsen. Jedes Symbol steht für einen Topf. Die Regressionsgeraden der mit Glycin behandelten Töpfe sind dargestellt: *A. capillaris* unfruchtbar (Steigung = 1.28, r^2 = 0.99) und fruchtbar (Steigung = 0.86, r^2 = 0.88); *H. lanatus* unfruchtbar (Steigung = 1.08, r^2 = 0.98); *L. perenne* unfruchtbar (Steigung = 1.52, r^2 = 0.87).

cant differences between species in either soil (Fig. 1B). A comparison between the two soils shows that mean shoot biomass over all species was significantly greater in improved compared with unimproved soil while changes in root biomass were only marginal (Tab. 1). Species did not differ significantly in their mycorrhizal colonization when considered for the soils separately (Fig. 1A). However, mycorrhizal colonisation of all grasses was significantly greater in unimproved than improved soil (Tab. 1).

3.2 Isotope analysis

We found a significant enrichment for both isotopes in root and shoot material of all plants grown in both soils (Fig. 1C and 1D), indicating direct glycine uptake. The uptake of stable isotopes showed a similar picture for shoots and roots (Fig. 1C and 1D). Mean uptake in percent of total isotope application ranged from 1.4/1.2 % in the unimproved soil to 7.1 / 2.2 % in the improved soil for ^{15}N /^{13}C, respectively. For plants grown on soil collected from the improved site, we found no significant species specific differences for ^{15}N or ^{13}C excess above natural abundance in either shoot or root material. In the unimproved site, however, the uptake of labeled glycine was significantly lower in *L. perenne* compared to *A. capillaris*, and this difference could be detected in both root and shoot material. *H. lanatus* was intermediate between the other two species without significant differences to either one of them (except for ^{15}N content in shoots that was higher than in *L. perenne*), thus forming a rank order with *A. capillaris* > *H. lanatus* > *L. perenne* that is most obvious in the stable isotope content of roots.

According to *Näsholm* et al. (1998), however, direct uptake of amino acids can be more accurately demonstrated by a significant relationship between ^{13}C and ^{15}N excess. For plants treated with ^{15}N-$^{13}C_2$-labeled glycine, the relationship between moles ^{13}C in excess and moles ^{15}N in excess was significant only in unimproved soil for *A. capillaris* (P = 0.002) and *H. lanatus* (P = 0.012), while for *L. perenne* in unimproved (P = 0.068) and *A. capillaris* in improved soil (P = 0.064) only marginal relationships could be found (Fig. 2). This provides strong evidence for direct uptake of intact amino acids in unimproved soil. When integrated across plant species, uptake of ^{15}N was greater in the improved than the unimproved soil (Tab. 1).

4 Discussion

The enrichment of plant material with both ^{13}C and ^{15}N after the addition of labeled glycine is a first indication of direct uptake of glycine (*Lipson* and *Monson*, 1998; *Lipson* et al., 1999). Therefore, this experiment provides some evidence that the three grasses commonly occurring in agricultural grasslands are able to take up organic N directly in the form of glycine. Mean uptake of 1.2–7.1 % of isotopes applied correspond well to earlier measurements, where some 3.5–12 % have been reported for similar experiments (*Lipson* and *Monson*, 1998; *Hodge*, 2001).

Our data demonstrate inter-specific variability in the uptake of organic N by the three grasses tested, but only in the less fertile soil of low productivity grassland. When grown in this soil, plant material of *A. capillaris* that commonly inhabits low productivity grassland, was significantly more enriched in both ^{13}C and ^{15}N than was shoot and root material of *L. perenne*, which typically dominates high fertility grasslands. ^{13}C and ^{15}N enrichment of *H. lanatus*, a common species on soils of intermediate fertility, was between these two species. Further evidence for the uptake of intact glycine and of inter-specific variability in organic N uptake in the less fertile soil came from the examination of linear relationships between the enrichment of ^{13}C and ^{15}N in plant tissue (*Näsholm* et al., 1998, 2000; *Nordin* et al., 2001). We found significant linear relationships only for two of the grasses, *A. capillaris* and *H. lanatus*, and only when these plants were grown in the low fertility soil. For *L. perenne* in unimproved soil and *A. capillaris* in improved soil, regressions were only marginal. These data suggest that the fraction of uptake of intact amino acid

was greatest for *A. capillaris* and *H. lanatus* when grown in the low fertility soil. Together, these findings provide first support for our hypothesis that typical grasses of low productivity grassland (i.e., *H. lanatus* and *A. capillaris*) are better able to take up amino acids than the dominant species of productive grasslands (*L. perenne*), but only when grown in low fertility soils (*Rodwell*, 1992).

Apart from an indication for direct uptake, the linear relationship between ^{15}N and ^{13}C excess is also a conservative estimate of the fraction of N taken up as intact glycine (*Näsholm* et al., 1998, 2000). For glycine-2-^{13}C-^{15}N, a slope of 1.0 would indicate that 100 % of the N was taken up as intact amino acid. However, our regression slopes range from 0.86 to 1.52. Most studies, so far, found clearly lower slopes than 1 (*Näsholm* et al. 2000, 2001; *Bardgett* et al. 2003), except for *Nordin* et al. (2001), who found an increased enrichment of ^{13}C in extracts of soluble N of *Picea* roots. The authors argued that a part of glycine-derived ^{15}N might have been transported towards the shoot or as exudates to the soil. This is in line with our finding of clearly more ^{15}N than ^{13}C in shoot material (Fig. 1). *Nordin* et al. (2001) further suggest a mechanism related to the glycine metabolism in microorganisms, which is primarily through the glycine decarboxylase pathway, forming serine, ammonia, and carbon dioxide from two glycine molecules (*Oliver*, 1994). The serine would result in a regression slope of 3.0, but only if ammonia was transported simultaneously to the shoot. However, both explanations are highly speculative and further studies are needed to analyze glycine metabolism prior to or following uptake.

When averaged over species, uptake of ^{15}N from glycine and total shoot growth were significantly greater in the improved than in the less fertile soil. In contrast to the low fertility soil, however, there was no evidence of inter-specific variability in uptake of organic N by the three grasses. The absence of significant relationships between enrichment of ^{13}C and ^{15}N for all grasses suggests that the primary pathway of ^{15}N uptake by plants in this soil was as glycine-N that had been mineralized by soil micro-organisms. This is consistent with previous field studies that show that most amino acids in soil are rapidly mineralized by soil microbes and consequently uptake of mineral N is the primary pathway of N acquisition in these systems (*Bardgett* et al. 2003). This pattern is clearly different from the unimproved soil, where direct uptake of amino acids was more evident in the two grass species that commonly inhabit these relatively infertile soils, and where differences among species in amino acid uptake were detected. These findings lead to the suggestion that, when grown in fertile soils, there is little difference in the ability of these grasses to acquire organic N. In contrast, in unimproved soil that is more N-limited, mineralization of organic N inputs is likely to be slower. Hence, inter-specific variation in the ability of plants to use organic N may become more evident (*Bardgett* et al., 2003). In the latter situation, differences in the ability of plants to acquire organic N directly may be a factor influencing the competitive interactions of these grass species, and may partly explain the increased dominance of species such as *A. capillaris* and the disappearance of *L. perenne* in low productivity grassland. In making these conclusions, however, we urge an element of caution; differences in uptake of glycine recorded between soils may also be related to physical characteristics of the soils that influence the diffusion of glycine through soil to the root zone (e.g. moisture content).

For all grasses, colonization of roots by arbuscular mycorrhizal fungi was greater in the unimproved soil than in the improved soil. This finding is consistent with previous studies that suggest that arbuscular mycorrhizal associations are more prevalent in unfertilizsed, semi-natural grasslands than in their highly fertilized counterparts (*Read* and *Haselwandter*, 1981; *Bardgett* et al., 1997; *Donnison* et al., 2000), due presumably to the sensitivity of these fungi to applications of inorganic fertilizers, particularly phosphate (*Sparling* and *Tinker*, 1978). It is known that these fungi can take up some amino acids directly (*Hawkins* et al., 2000), so it is possible that they were instrumental in the direct uptake of organic N in this study. In contrast, *Hodge* (2001) found that arbuscular mycorrhizal colonization did not affect organic N uptake of *Plantago lanceolata L.* seedlings. It is important to acknowledge that we cannot explicitly test for the role of these fungi in the uptake of organic N in the present study, since no manipulations of the mycorrhizal status were made. Our data point to a possible role for these fungi in amino acid uptake, especially in low fertility soils where they are more abundant and where direct uptake of glycine by grasses was more clearly detected. In contrast, however, similar mycorrhization of all three grasses resulted in lower amino acid uptake for *L. perenne*. Therefore, any presumed relationship between mycorrhizal infection and amino acid uptake would need to be species specific. Further studies are needed to examine the role of mycorrhizal fungi in organic N uptake in these grassland soils.

In conclusion, our data indicate that grasses are able to take up organic N directly, especially in low fertility soils, where inter-specific variability in uptake by grass species is evident. Grass species that typically dominate the plant community of low productivity grassland are better able to utilize amino acid N inputs than *L. perenne*, which typically dominates high fertility grassland. Greater colonization of roots by mycorrhizal fungi in low fertility soils may be instrumental in the direct uptake of amino acids, but further studies are needed to test this claim. Overall, our findings provide some explanation for the increased competitive dominance of slow-growing species like *A. capillaris*, and the demise of fast-growing species such as *L. perenne*, in low fertility situations where amino acids are the dominant form of soluble N (*Bardgett* et al., 2003).

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