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Arbuscular Mycorrhizal Symbiosis and Phosphorus Nutrition: Effects on Amino Acid Production and Turnover in Leek

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Abstract

This study examines leek (*Allium porrum* L.) plants colonized by the arbuscular mycorrhizal fungus *Glomus etunicatum* Becker & Gerdemann under conditions where the symbiosis is actively affecting host plant growth and carbon metabolism. Isotopic labeling with ^{15}N nitrate together with amino acid analysis and mass spectrometry was used to assess the free amino acid pools of root and shoot tissues in colonized plants (M) compared to uncolonized plants grown either with (NM+P) or without (NM–P) supplemental phosphate. Total amino acid levels in roots were lower in M than in NM–P plants and this effect also was induced partially in NM+P plants by supplemental phosphate. The relative proportions of different amino acids were not affected by the AM symbiosis nor were amino acid levels in leaves affected by either colonization or increased phosphate supply. Fractional labeling was substantial in all free amino acids after a six-week labeling period and enrichments were higher on average in M than in NM–P. There were no indications of effects of mycorrhizas upon relative enrichments in different amino acids. However, a higher

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incorporation of label was found in Asp, Glu, and Gln in short term ^{15}N labeling experiments (72 h) with M than NM–P plants. The average labeling in all amino acids was higher in the roots of M plants. More rapid turnover of amino acids was noted in the M plant roots, however this was probably due to the lower concentration of amino acids found in the plant. These results suggest that there are indeed effects of AM symbioses on overall nitrogen handling by the host but that these are secondary effects of the improved phosphate supply to the host and do not cause significant shifts in levels or fluxes through different amino acids.

Keywords: Leeks, *Glomus etunicatum*, mass spectrometry, ^{15}N, amino acids, labeling

1. Introduction

Arbuscular mycorrhizal (AM) symbiotic fungi have been an integral part of the life of land plants for over 400,000,000 years (Remy et al., 1994), and they are the most widespread both geographically and ecologically (Smith and Read, 1997). Among the wide variety of benefits to the host plant that have been ascribed to the AM symbiosis are: resistance to plant pests (Hooker et al., 1994), improved water relations (Davies et al., 1993; Subramanian and Charest, 1998, 1999), increased growth and yield (Mosse, 1973), and increased nutrient uptake (George et al., 1995).

The effects of AM fungus colonization on the host in many cases have been shown to be benefits of the enhanced phosphorous status of mycorrhizal plants (Smith and Reed, 1997). The extraradical hyphae of the fungus take up phosphorous from the soil and transfer much of it to the host (Bolan, 1991; Harrison and van Buuren, 1995). This has been shown for Zn and Cu which are also immobile in the soil solution (Li et al., 1991; Burkert and Robson, 1994).

The role of AM fungi in nitrogen uptake and metabolism of plants is less understood, however (George et al., 1995). Field studies have shown little if any direct effect of AM fungus colonization on the nitrogen status of host plants, which contrasts with findings for Ericoid and ectomycorrhizae (Marshner and Dell, 1994). On the other hand, a number of studies using ^{15}N labeled forms of nitrogen have demonstrated that the fungal symbiont is able to take up at least modest amounts of nitrogen and transfer it to the host (Ames et al., 1983; Frey and Schüepp, 1993; Johansen et al., 1996). Transfer of ^{15}N label from one host to another via the AM fungus colonizing both has been demonstrated (Bethlenfalvay et al., 1996). The extraradical hyphae of AM fungi are capable of assimilating ammonia and to a lesser extent nitrate (Johansen et al., 1996; Bago et al., 1996), except under certain water stress conditions (Tobar et al., 1994), and so the potential exists for uptake of nitrogen by AM fungi and for its transfer to the host plant.

Two aspects need further investigation to reconcile these observations: (i) the efficiency with which AM fungi can take up, metabolize and transfer different forms of organic and inorganic nitrogen to the host; and (ii) the direct and indirect effects of AM fungus colonization upon regulation of nitrogen metabolism in the host. Mindful of the problems associated with the relatively high mobility of nitrogen in the soil and the potential for interconversion of different nitrogen forms, we adopted a dual approach to addressing these questions. In the present study we sought to assess the effects of AM fungi on nitrogen metabolism in the host by supplying ^{15}N labeled nitrate to mycorrhizal and nonmycorrhizal plants and measured concentrations and percentage labeling of free amino acids in the host tissue. Growth conditions were chosen where the AM symbiosis was actively promoting plant growth due to enhanced phosphorous uptake, but where most nitrogen was likely to be taken up directly by the host roots. In a separate study (Rolin et al., 1997) we used the *in vitro* dual culture system (St-Arnaud et al., 1996; Villegas et al., 1996) to rigorously test the efficiency of the AM fungus to take up, metabolize, and exchange nitrogen with the host.

2. Materials and Methods

Growth of plants

Allium porrum L. cv Musselburgh plants were grown as previously described (Shachar-Hill et al., 1995). Briefly, plants were germinated from seed and transplanted into conical plastic pots ('Super-cell C-10', Stuewe and Sons, Corvallis, OR, USA) (approx. 20 cm long, total volume 165 cm^3) containing a sand/calcined clay mix (1:1 [v/v]). Inoculum of *Glomus etunicatum* Becker & Gerdemann (isolate 8961 from Native Plant Industries, Salt Lake City, UT, USA) was incorporated into the potting mix to generate colonized plants and omitted from uncolonized controls. Plants then were grown for four and a half months in a greenhouse under natural photoperiods during which roots grew throughout the pot.

Two experiments were conducted. Three groups of four plants were used for a long-term labeling experiment: one group was inoculated with *G. etunicatum* and the other two groups were nonmycorrhizal. Plants were fertilized weekly with Hoagland's solution (Hoagland and Arnon, 1938) containing either 0.1 mM phosphate (mycorrhizal plants [M] and low-P nonmycorrhizal plants [NM-P]) or 1 mM phosphate (high phosphate nonmycorrhizal plants [NM+P]). Nitrate in the fertilizing solutions was enriched to 41.1% in ^{15}N during the 6 week labeling period which commenced after four and one half months of growth.

A short-term pulse-chase experiment also was conducted. Fifteen

mycorrhizal and 15 non-mycorrhizal plants were grown with weekly fertilization with Hoagland's solution with 0.1 mM phosphate and then deprived of fertilization for 4 weeks before the labeling period to reduce unlabeled nitrogen in the soil. Three plants from each group were harvested (t_0) and labeling solution (Hoagland's solution with all the nitrogen labeled as ^{15}N) was added to the remaining plants. Three more plants from each group were harvested after 24 hours and the label was washed out of the remaining pots with 5 pot volumes of water. Three plants from each treatment then were harvested at days 2, 4 and 8. Fertilization of remaining plants occurred at days 3 and 5.

Sample preparation and analysis of amino acids

At harvest, plants were removed from pots and their roots washed free of soil. Root systems were divided into three sections: top (2–3 cm), midsection (approximately 5.5 cm) and bottom (3–4 cm). The fresh weights of roots and shoots were determined. Several root segments were taken for analysis of total colonization and the presence of arbuscules as previously described (Shachar-Hill et al., 1995). The remaining root and shoot tissues were frozen separately on dry ice and lyophilized. The tissues then were ground finely with a mortar and pestle and 100 mg of each sample was extracted at 85–90°C once with 6 ml of 80% ethanol and twice with 6 ml of 60% ethanol. Samples were centrifuged following each extraction and supernatants were pooled. The solutions then were passed through a Biorad 50-X8 resin and amino acids were eluted with 6 N ammonium hydroxide, acidified, and evaporated to dryness.

Amino acid analysis

The most abundant amino acids in extracts were determined using a Waters Pico-Tag amino acid analyzer using the Pico-Tag method. The threshold for detection of amino acids in standard solutions was 30 picomoles of each amino acid per assay, corresponding to approximately 10 nanomoles per gram dry weight of tissue in the extracts.

Mass spectral amino acid analyses

Mass spectrometric analyses of ^{15}N labeling of amino acids were performed on the lyophilized extracts after incubation for 45 min at room temperature in pyridine: N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTAF) to yield silated derivatives. Analysis of amino acids was performed on a Hewlett Packard series 5890 gas chromatograph equipped with

a splitless injector, open -tubular column of 0.25 µm-thick BP-15 film (0.18 mm i.d. H 0.4 mm o.d. H 30 mm long, SGE Inc.) as described by Tjeerdema et al. (1991); interfaced to a 5972 mass selective detector. Zone temperatures were: injector, 260°C: column held at 60°C for 1.5 min after injection, ramped up to 150°C at 20°C /min, then to 300°C at 6°C/min, where it was held for 5 min; and detector, 320°C. The He carrier gas velocity was 36 cm/sec. Identities were confirmed by gas chromatography-mass spectrometry of authentic standards. The presence of ^{15}N labeling was detected in the mass spectra of the derivatized amino acids by comparing the intensities of ions having masses that differ by one or two mass units from the M-57 ion of the derivatized amino acid. Correction for background contributions to higher mass ions was made from the known C and Si isotopic natural abundances and confirmed with unlabeled standards.

Because of potential inaccuracies in such measurements, and because some of the differences that we observed in isotopic enrichments were rather small, we sought to verify the reliability of measuring fractional isotopic labeling of amino acids by chromatographic mass spectrometry in leek extracts. Consistently close agreement (always better than 95% percent and better than 99% for more than 3/4 of the repetitions) was obtained between the mass distributions expected on the basis of natural abundance isotope levels and those for all the amino acids we observed in mass spectra of unlabeled amino acid mixtures and extracts of unlabeled plant tissues (data not shown). The estimation of fractional enrichments was further checked on a range of mixtures of unlabeled and ^{15}N-enriched glycine. The expected and calculated mass distributions for these mixtures (0,10,25,50,75 and 100% enriched in ^{15}N) agreed to within 5% in all cases and for the majority of spectra to within 2%, which is less than the estimated potential errors introduced by weighing and pipetting. Fractional labeling was not determined for several amino acids in all samples generally due to low levels and/or substantial chromatographic overlaps.

3. Results and Discussion

AM fungus colonization and its effects on growth

The percentage of root length colonized by the AM fungus and the percentage of root length in which arbuscules were present were determined for the mid-sections of the root system for each plant. The total colonization among the colonized plants varied from 14 to 64% (mean 40.5%, S.D 10%) and the occurrence of arbuscules ranged from 5% to 48% of the root length. No colonization was detected in any of the roots from uninoculated plants. Colonization was substantially higher in the midsection than in the top and

bottom portions of the root systems of leeks grown under these conditions (data not shown). Because of this and to compare results of amino acid analyses with our previous observations on carbohydrate metabolism in such plants, in which we used midsections of the root systems, the top and bottom parts of each root system were combined and the midsection analyzed separately.

The ratio of the fresh weight of the shoots to that of the whole roots for each plant (shoot:root ratio) was taken to assess the effects of colonization and phosphorous supply on growth. The ratios (mean±SEM) for the M, NM–P and NM+P groups were 0.86±0.04, 0.66±0.08 and 0.72±0.14, respectively. The shoot-to-root ratio for the mycorrhizal plants was significantly (P=0.05) higher than that of the NM–P group. The intermediate value of the NM+P group (not significantly different from the M group) indicated that the effect of mycorrhizal fungus colonization upon growth was partially or wholly due to improved phosphate nutrition. This was consistent with improved phosphorous uptake by colonized plants reported for a wide variety of plant and AM fungal species (Smith and Reid, 1997).

Levels of free amino acids in tissues of colonized and uncolonized plants

The concentrations of the free amino acids in plant tissues reflected the balance between primary metabolic fluxes, protein turnover, storage and transport. Significant changes in the balance of these may therefore be reflected in alterations of absolute or relative levels of the different free amino acids. Levels of amino acids are shown in Fig. 1 for the more abundant amino acids in root midsection (Main) (1A), root top and bottom (1B), and leaves (1C). The most prevalent amino acids in root tissues were asparagine, arginine, glutamine, glutamate and aspartate, whereas arginine predominated in shoots. There was no statistically significant difference in the level of any one amino acid due to either AM colonization or increased phosphate supply. However, it may be seen from Figs. 1A and 1B that, for each amino acid where a comparison is possible (9 amino acids in the root midsections and 13 in the top/bottom root samples), mean levels were higher in NM–P plants than in M plants, with the NM+P generally having intermediate values. This difference in overall amino acid levels between M and NM–P roots was statistically significant. The fact that the NM+P group has intermediate levels of amino acids suggests that some of the reduction in amino acids, perhaps all, was due to increased phosphate supply in the mycorrhizal plants (Nemic and Meredith, 1981). While there was an overall decrease in amino acid levels in roots in M plants, there was no indication that the relative contribution of each amino acid is affected by AM fungus colonization or by phosphate supply (data not shown). A comparison of the data for each amino acid in Fig. 1A with those in 1B, gave

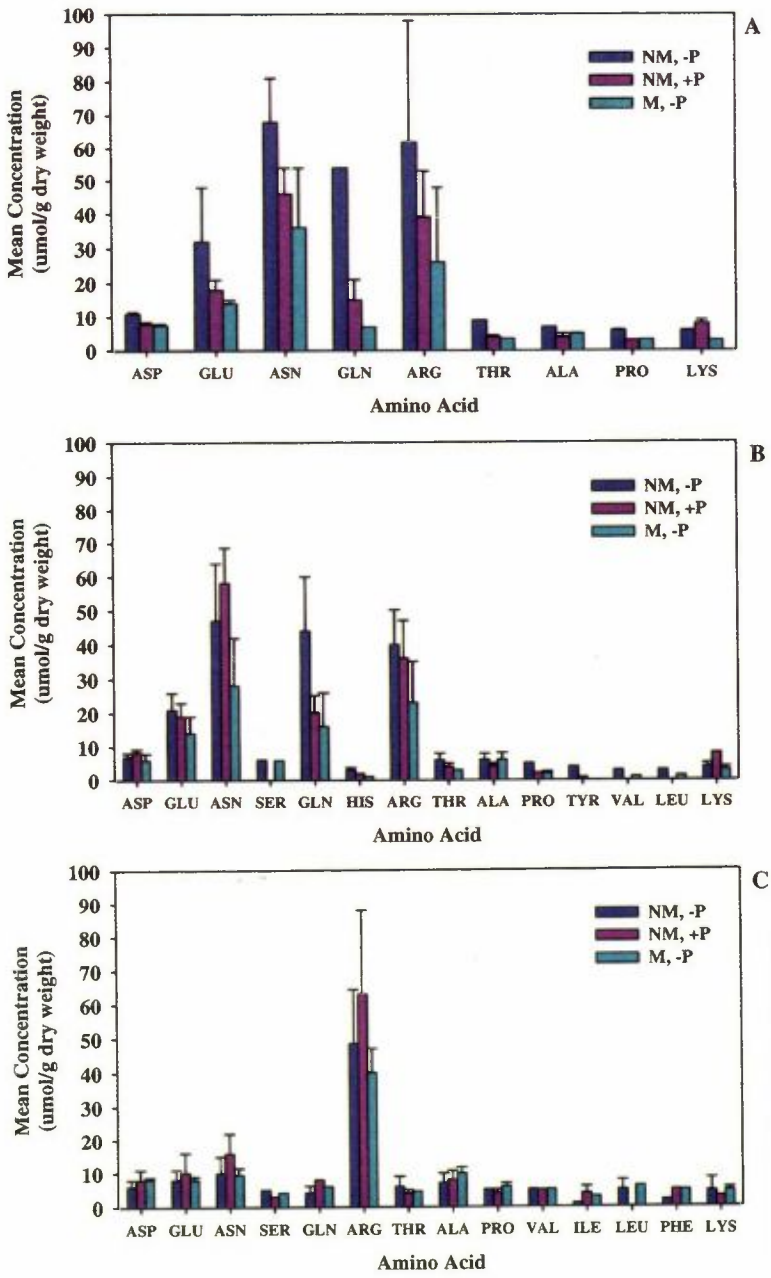

Figure 1. Amino acid concentrations found in the A) main or mid section of roots, B) root top and bottom sections, and C) shoots of mycorrhizal and non-mycorrhizal leeks following four and one half months of growth with weekly fertilization with Hoagland's solution containing either 0.1 mM phosphate (mycorrhizal plants and low –P plants) or 1 mM phosphate (high phosphate non-mycorrhizal plants).

Table 1. Percent ^{15}N labeling in amino acids of the midsections of leek roots

Amino acids	% ^{15}N label in M root midsections	S.D.	% ^{15}N label in NM+P root midsections	S.D.	% ^{15}N label in NM−P root midsections	S.D.
Alanine	30.74	2.29	31.26	3.69	29.93	5.90
Glycine	24.88	3.54	25.29	1.37	23.73	3.40
Valine	30.53	2.21	28.01	2.02	26.15	3.68
Leucine	34.99	1.86	31.70			
GABA	40.36		29.78		18.13	
Proline	27.62	3.36	20.37			
Methionine	35.69					
Serine	26.92	2.72	27.42	2.52	26.64	4.60
Threonine	26.72	3.04	24.58	3.15	22.69	2.67
Phenylalanine	28.68		25.71	7.10		
Aspartic acid	35.07	3.66	33.97	2.57	32.39	1.53
Cysteine						
Glutamic acid	37.18	3.18	36.10	1.93	34.10	1.83
Asparagine	47.44	20.90	56.14	4.06	53.97	4.54
Lysine	44.56		38.08	4.20		
Glutamine	65.02		58.73	1.50	68.40	
Histidine	38.74	3.01	36.35	8.76	34.57	12.80
Tryptophan	47.59	22.58	35.55	7.37	33.64	8.03
Tyrosine	24.46	5.66	25.52	6.69	16.45	11.40

no indication that AM fungus colonization affects the relative distribution of amino acids within the roots – the much more highly colonized root midsections had levels of each amino acid similar to those in the less colonized parts of the root.

In contrast with the observations on levels in root tissues, there was no indication that levels or proportions of free amino acids in leaves are affected by phosphate supply or by the AM symbiosis (Fig. 1C). Arginine represented close to half the total detected free amino acid pool in each treatment.

We next sought to assess the fluxes through the free amino acid pools by analyzing the fractional levels of labeling in the different amino acids of the various plant tissues (Tables 1–3). A comparison among colonized and uncolonized plants with or without supplemental phosphate showed similar labeling levels in each amino acid. As with amino acid levels, the fractional labeling in each amino acid considered separately was not statistically different among treatment groups, but averaged across all amino acids there

Table 2. Percent ^{15}N labeling in amino acids of the top/bottom sections of leek roots

Amino acids	% ^{15}N label in M root top/bottom	S.D.	% ^{15}N label in NM+P root top/bottom	S.D.	% ^{15}N label in NM–P root top/bottom	S.D.
Alanine	35.40	3.76	33.46	1.98	29.78	3.39
Glycine	22.78	0.68	29.01	2.09	28.79	4.08
Valine	32.52	2.86	32.10	2.18	29.17	1.41
Leucine	34.17	1.99	33.85	1.63		
GABA			44.17	1.37		
Proline	27.90		25.48			
Methionine	34.51					
Serine	31.00	2.34	30.76	1.82	30.25	4.31
Threonine	25.97	3.10	27.24	3.57	25.17	9.26
Phenylalanine	27.29		26.63	4.88	29.17	
Aspartic acid	34.00	3.84	34.45	3.74	30.15	2.62
Cysteine						
Glutamic acid	35.77	4.64	36.81	0.98	32.99	1.69
Asparagine	56.24	2.89	58.86	4.89	58.17	7.94
Lysine	42.78		42.55	1.88	36.12	
Glutamine	57.41	7.80	58.65	2.04	56.46	1.49
Histidine	37.95	0.84	36.82	7.30	31.99	
Tryptophan	35.20	1.36	36.63	7.46	29.91	1.29
Tyrosine	25.15	2.47	28.50	3.91	25.10	5.02

were small, significant differences among treatments in the amino acids of the roots.

The NM–P group had lower levels of labeling than the M plants. Levels of labeling in amino acids in the root midsections (Table 1) and top/bottom samples (Table 2) of NM-P plants were 92±2% and 94±2% of those in the M group (mean±SEM for all amino acids, excluding any for which the coefficient of variation was more than 30%). Amino acids in the NM+P midsections were on average labeled to 98±2% (Table 1) and top and bottom, 102±1% (Table 2) of the M group levels. Thus mycorrhizal colonization or P supplementation resulted in a slightly higher labeling level in the host plant tissues, indicating that free amino acid pools may turn over faster on average than pools in non-mycorrhizal plants lacking supplemental P.

Substantial labeling levels were reached in all free amino acid pools of leaf and root tissues in all treatment groups and it might be argued that nitrogen labeling levels had reached equilibrium so that the measurements would be

Table 3. Percent ^{15}N labeling in amino acids of leek leaves

Amino acids	% ^{15}N label in M root leaves	S.D.	% ^{15}N label in NM+P root leaves	S.D.	% ^{15}N label in NM–P root leaves	S.D.
Alanine	34.00	2.43	31.22	3.21	31.25	2.15
Glycine	27.63	5.30	29.44	3.45	27.84	1.49
Valine	29.76	3.73	28.87	8.12	28.10	2.59
Leucine	31.24	3.53	31.06	3.49	31.12	1.91
GABA	40.21	7.15	36.36	3.38	31.31	5.71
Proline	35.00	8.50	26.17	8.49	29.57	5.63
Methionine	31.60	2.89	29.00	2.60	30.83	3.33
Serine	29.31	4.53	30.56	3.72	29.30	2.58
Threonine	26.69	6.25	27.48	1.76	27.67	2.16
Phenylalanine	28.34	5.31	26.53	3.62	24.19	2.14
Aspartic acid	31.65	7.55	33.59	1.98	32.20	2.69
Cysteine						
Glutamic acid	33.06	3.25	32.42	2.68	33.58	2.84
Asparagine	53.17	2.32	54.51	3.00	51.99	2.36
Lysine	41.42	2.51	39.16	6.10	37.77	3.83
Glutamine	54.06	1.32	35.97	28.3	53.88	1.85
Histidine	41.59	3.43	42.96	4.42	41.85	2.04
Tryptophan	33.53	1.92	33.90	5.25	35.16	3.73
Tyrosine	30.65	7.61	20.83	7.73	23.05	7.04

insensitive to any specific differences in nitrogen fluxes due to the AM symbiosis or phosphate supplementation. Two observations suggest that this is not the case and that the similarities between groups reflect both similarities in relative nitrogen fluxes through different amino acid pools and differences in overall total amino acid turnover rates in the tissues of the three groups. First, enrichment of ^{15}N in the fertilizer solution was 41.1% and the levels reached are significantly less than this in root tissue for all free amino acids that contain one nitrogen atom. (Amino acids with two nitrogens per molecule consequently have higher levels of ^{15}N). This showed that full isotopic equilibration of free amino acid pools had not taken place during the six-week labeling period. This disequilibrium reflects the fact that while amino acids synthesized during the labeling period will be labeled to levels close to 41%, any free amino acids that had been synthesized previously or that were generated from turnover of unlabeled proteins, would not be labeled. Second, this balance appears to be different for different amino acids with the same

number of nitrogen atoms so that for example labeling in Lys>Gln, Asn; and Glu,Asp,Ala>Gly,Phe,Tyr. We conclude from these observations that the patterns of nitrogen fluxes through the pools of free amino acids in root and shoot tissues are very similar in AM colonized or phosphate fertilized plants compared to uncolonized unfertilized controls.

Short-term labeling experiment

To look for any short-term differences in N uptake and metabolism, we next followed labeling in M and NM–P plants over a pulse-chase period of 8 days (see Materials and Methods). The average level of labeling in all amino acids detected in roots is shown in Fig. 2. Mycorrhizal roots reached higher ^{15}N levels over the whole timecourse but variation between labeling in different amino acids prevented these differences from being statistically significant for any one time point. If the most highly labeled amino acids (Asp, Gln and Glu for both groups of plants at all time points) were considered, the difference between M and NM–P was clearer. The time course of labeling in Glu (Fig. 3) is representative of that of Asp and Gln (data not shown). Labeling of Glu in M roots rose faster during the 24 h ^{15}N labeling period than in NM–P roots, and decreased to similar values by day 4 (72 h of chase). Thus amino acids in M roots apparently turn over faster than in NM–P roots under these conditions and this is perhaps not surprising considering the smaller concentrations of free amino acids. Given the effects of P supplementation above, we suggest that the differences in root amino acid turnover rates are also a secondary effect of improved host P nutrition in this system. The labeling levels in leaves of these plants showed similar trends (data not shown), with Asp, Glu and Gln being the most highly labeled and rising at day 1 and falling thereafter. The variation in levels of labeling from plant-to-plant was larger than for labeling in roots and there were no significant differences between M and NM–P groups.

We know that alterations in growth in this active AM system are mimicked by increased phosphorous fertilization. However, our observations on root carbon metabolism (Shachar-Hill et al., 1995) indicate that supplemental phosphate does not mimic the effects of colonization on that aspect of physiology. Our results here demonstrate that the host plant N metabolism also is altered by the symbiosis, relative to that of an uncolonized plant, but that these effects are secondary to and may be the result of differences in phosphorous nutrition.

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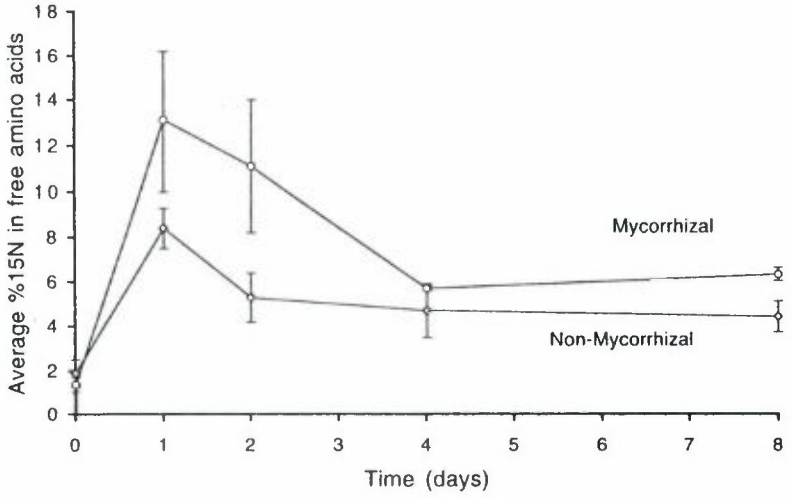

Figure 2. Time course of average ^{15}N incorporation and turnover in free amino acids of (o) leek roots colonized with *G. intraradices* [M] following labeling with $^{15}NO_3$ for 24 hours, and subsequent washout with 5 pot volumes of water; (◊) non-colonized leek roots without phosphorus [NM–P] following labeling with $^{15}NO_3$ for 24 hours, and subsequent washout with 5 pot volumes of water. Each point is the mean (±SEM) of three independent replicates.

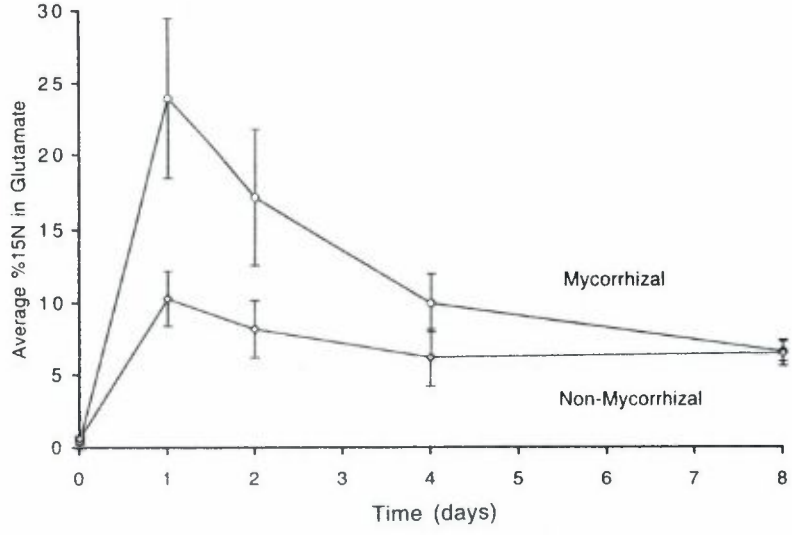

Figure 3. Time course of average ^{15}N incorporation and turnover in glutamate of (o) leek roots colonized with *G. intraradices* [M] following labeling with $^{15}NO_3$ for 24 hours, and subsequent washout with 5 pot volumes of water; (◊) non-colonized leek roots without phosphorus [NM–P] following labeling with $^{15}NO_3$ for 24 hours, and subsequent washout with 5 pot volumes of water. Each point is the mean (±SEM) of three independent replicates.

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