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Nitrogen mobilization and nutrition in ectomycorrhizal plants

Introduction

Constraints upon plant growth imposed by low availability of N are a characteristic feature of many ecosystems dominated by ectomycorrhizal (ECM) plants. Thus, in the extensive boreal (Tamm, 1991) and temperate (Ellenberg, 1988) forests of the northern hemisphere the accessibility of N is the most important determinant of productivity. Our understanding of the N dynamics of these systems has been improved by the recognition that the forms in which the element may be available to ECM plants in forests are diverse. Despite the early suggestion of Frank (1894) that ECM colonization may provide plants with access to organic forms of N, the view that mineral N was the only important source of the element predominated during much of the following century. Re-emphasis of the potential importance of organic N sources for the nutrition of mycorrhizal plants came from studies first of ericoid mycorrhiza (ERM) (Bajwa *et al.*, 1985; see Chapter 11) and then of ECM symbioses (Abuzinadah and Read, 1986a, 1986b; Read, 1991a; Bending and Read, 1995a, 1995b; Chalot and Brun, 1998; Emmerton *et al.*, 2001a, 2001b; Nasholm and Persson, 2001). By stressing that the activities of the fungal symbionts might provide plants with access to forms of N which would otherwise be completely unavailable to them, these studies led to a resurgence of interest in the roles of organic N in plant nutrition. The result has been that there is now a more balanced appreciation of the spectrum of N sources likely to be involved in the nutrition of ECM plants and of the environmental circumstances under which they are likely to be the predominant available forms. Attention is now being given to the fundamental molecular and biochemical events associated with the mobilization, assimilation and transport of all potentially available forms of N and the improved understanding of the environmental constraints is helping to place these processes into particular ecological contexts.

Use of N by ectomycorrhizal fungi in pure culture

Inorganic N sources

The ECM fungi that have been successfully grown in axenic culture are similar to other fungi in the kinds of N compounds that they can use for growth. The early workers (Norkrans, 1950; Rawald, 1963; Lundeberg, 1970) found a range of relative abilities to use ammonium and nitrate among the species and strains of ECM and saprotrophic fungi that they studied (see Harley and Smith, 1983 for details). Nothing was discovered which distinguished ECM fungi as a group and later work confirmed these observations (France and Reid, 1984; Littke *et al.*, 1984; Genetet *et al.*, 1984; Plassard *et al.*, 1991). Most of them grow fastest on ammonium and some can use nitrate but others not. Experimental work on N absorption is beset with difficulties concerning pH change, which can be very significant in the unbuffered media often employed. Absorption of ammonium results in a marked lowering of the pH, which may be followed by a sharp cessation of growth. The form of N present in the medium is strongly affected by pH. The pKa for protonation of ammonia is 9.25, with the result that ammonium rather than ammonia will be the predominant form in most culture media and under most growth conditions. This has implications for the membrane transport processes likely to be involved in uptake. Absorption of nitrate, as well as the release of ammonium from amides or other readily hydrolysed compounds, causes an increase in pH which is usually slower and may have a less marked effect on growth than the reductions in pH. In spite of these problems, there is not much doubt of the broad results, although there is much variation between different species and strains of ECM fungi in requirements for inorganic N.

There is striking variability both between (France and Reid, 1984; Plassard *et al.*, 1986; Anderson *et al.*, 1999) and within (Ho and Trappe, 1987; Anderson *et al.*, 1999) ECM fungal species with respect to their abilities to use nitrate. Among those ECM fungi that most readily use nitrate are members of genera like *Hebeloma* which, as pioneer occupants of disturbed habitats, are most likely to encounter this form of N (Marmeisse *et al.*, 1998). The nitrate nutrition of *H. cylindrosporum*, some strains of which achieve a 10-fold greater biomass on nitrate than on ammonium, has been extensively investigated (Plassard *et al.*, 1991, 1994). The *Km* for nitrate uptake in this fungus of 67 μM (Plassard *et al.*, 1994), compared with 12 μM for *Rhizopogon roseolus* (Gobert and Plassard, 2002), indicates the large interspecific differences between fungi in their affinity for nitrate. In the latter fungus, nitrate uptake occurred at the same rate regardless of whether the mycelium had been pretreated in 0.05 mM nitrate or not, suggesting that no inducible nitrate transporter exists in this organism. Jargeat *et al.* (2003) isolated a gene (*NRT2*) coding for a nitrate transporter from *H. cylindrosporum*. The polypeptide which it encodes is characterized by 12 transmembrane domains that are suggested to consist of a long possibly intracellular loop and a short C-terminal tail. Transcription of the *NRT2* gene was repressed by ammonium and stimulated under conditions of low exogenous supplies of both nitrate and simple sources of organic N.

Progress has been made towards characterization of the molecular events and physiological processes associated with nitrate assimilation. Genes encoding proteins involved are induced by nitrate and subject to N catabolite repression.

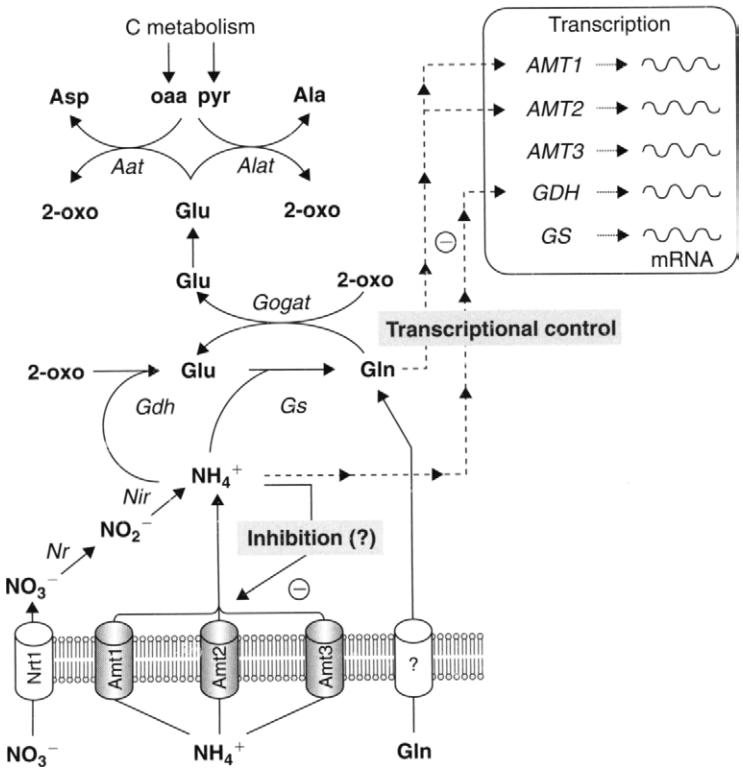


Figure 9.1 A model describing the regulation of nitrogen transport and assimilation in *Hebeloma cylindrosporum*. This ectomycorrhizal fungus is able to use nitrate, ammonium and amino acids as nitrogen sources. Under low ammonium status, the genes *AMT1*, *AMT2*, *AMT3*, *GDH* (*GDHA*) and GS (*GLNA*) are transcribed, which results in elevated ammonium uptake and metabolism capacities. Under ammonium excess, *AMT1*, *AMT2* and *GDHA* are efficiently repressed, which results in reduced ammonium assimilatory capacities. Under these conditions, *AMT3* and *GLNA* would ensure the maintenance of a basal level of ammonium assimilation. *AMT1* and *AMT2* transcript levels are controlled through the effect of intracellular glutamine, whereas the *GDHA* and *NAR1* mRNA levels are controlled by ammonium (dotted lines). Ammonium uptake activity may be controlled by intracellular NH_4^+ through a direct effect (dotted lines). *2-oxo* 2-oxoglutarate, *oaa* oxaloacetate, *pyr* pyruvate, *GOGAT glutamate synthase*, *Aat* aspartate aminotransferase, *Alat* alanine aminotransferase, *NR* nitrate reductase, *NiR* nitrite reductase, *Nrt1* nitrate transporter, *GAP1* general amino acid transporter. From Javelle *et al.* (2003b), with permission.

Two nitrate reductase genes have been cloned in *H. cylindrosporum* (Jargeat *et al.*, 2000). One (*nar 1*) has been transcribed and shown to code for a 908 amino acid polypeptide, while the other (*nar 2*) is considered to be an ancestral but non-functional duplication of *nar 1*. Above a basal level, transcription of *nar1* is repressed by high concentrations of ammonium, but strongly induced in the presence of nitrate or under low total N conditions. Transcription also occurs when organic sources such

as glycine constitute the sole sources of N in the bathing medium, indicating that an exogenous supply of nitrate is not necessarily required to induce transcription of the gene. *Nar1* gene expression in *H. cylindrosporum* thus appears to be regulated primarily by the availability of ammonium. These authors propose that the lack of a nitrate induction system may represent an adaptation to the forest soil environment in which many different N sources are present all at low concentrations.

Since ammonium is the major source of mineral N in most forest soils (Read, 1991a; Marschner and Dell, 1994), it is perhaps not surprising that the majority of ECM fungi preferentially use the ammonium ion when grown in culture. The kinetics and energetics of ammonium uptake have been examined in *Paxillus involutus* (Javelle *et al.*, 1999). K_m and V_{max} were 180 and 380 μM, respectively. Experiments carried out using both short-term (hours) and long-term (days) incubation indicated that ammonium uptake was strongly regulated by the presence of the organic N sources glutamate and leucine. This observation is of ecological interest in view of the frequent co-occurrence of amino compounds and ammonium in soils supporting ECM plants (Read *et al.*, 2004).

In common with other fungi, most of the N from ammonium is assimulated by ECM fungi into the amide group of glutamine by the glutamine synthetase (GS-GOGAT) pathway, or as the amino group of glutamate through the glutamate dehydrogenase (GDH) pathway. While both of these routes can be present, the relative importance of each differs according to fungal species.

The genes coding for ammonium uptake and transport have now been cloned and this has enabled progress towards understanding of both their function and regulation. The ammonium transporter gene *AMT1* has been cloned from *Tuber borchii* (Montanini *et al.*, 2002) and *H. cylindrosporum* (Javelle *et al.*, 2001, 2003a). This is a high-affinity transporter which is expressed only under conditions of N deficiency (Javelle *et al.*, 2003a). Further transporters, *AMT2* and *AMT3* have also been characterized from the latter fungus (Javelle *et al.*, 2001). One of these, *AMT3*, is a low-affinity transporter which is highly expressed but not highly regulated. In addition to the transporters, genes encoding both GS (*GLNA*) and one encoding GDH (*GDHA*) (see below) have also been cloned and characterized (Javelle *et al.*, 2003b, 2004). This work has enabled the construction of a model describing the uptake and regulation of ammonium transport in *H. cylindrosporum* (see Figure 9.1). When exposed to low levels of ammonium, the regulatory genes *AMT1*, *AMT2*, *AMT3*, *GLNA* and *GDHA* are transcribed, resulting in increased capacity for ammonium uptake and metabolism. Under conditions of elevated ammonium availability, *AMT1*, *AMT2* and *GDHA* are effectively repressed, reducing the ability of the mycelium to assimilate ammonium. In the latter situation, it is expected that the activities of *AMT3* and *GLNA* would ensure a basal level of ammonium assimilation. The model proposes that the transcript levels of *AMT1* and *AMT2* are controlled through the effects of intracellular glutamine, whereas the *GDHA* mRNA accumulation is controlled by ammonium (dotted lines in Figure 9.1).

The high-affinity *AMP1* ammonium transporter is a member of a gene family, *MEP2*, which is involved in mycelial proliferation in fungi and is essential for hyphal differentiation in yeast (*Saccharomyces cerevisiae*) (Madhani and Fink, 1998). On the basis that incorporation of the *AMP1* and 2 genes of *H. crustuliniforme* into *MEP2*-deficient *S. cereviseae* restored the ability of the yeast to produce pseudohyphae, Javelle *et al.* (2003b) have proposed that these ammonium transporters may

also act as ammonium sensors which stimulate hyphal proliferation in response to low levels of ammonium enrichment. Observations such as these indicate that genes identified as having a specific function in the context of nutrition may also have other roles, in this case as triggers of morphogenetic change. The possibility that expression of the two attributes takes place in concert to facilitate ammonium capture is particularly interesting.

Analysis of the major pathways of N metabolism in *Cenococcum geophilum* (Genetet *et al.*, 1984; Martin, 1985; Martin *et al.*, 1988a), *Hebeloma cylindrosporum* (Chalot *et al.*, 1991) and *Laccaria laccata* (Brun *et al.*, 1992) indicates that the GS pathway of ammonium assimilation predominates in these fungi. It was shown that GS represented about 3% of the total soluble protein pool of *L. laccata*, a value considerably greater than that for GDH, which accounted for only 0.15%. Using ^{15}N-labelled ammonium as tracer, up to 40% of assimilated ^{15}N was found in the amide group. The GS of *L. laccata* is known to have a very high affinity for ammonium, further suggesting that, in this fungus at least, GS is the main route of ammonium assimilation (Brun *et al.*, 1992). This may also be the case in many ECM fungi and would be consistent with earlier work showing that, in the presence of ammonium, dark fixation of $^{14}CO_2$ results in preferential incorporation of label into glutamine by excised *Fagus* mycorrhizas (Harley, 1964; Carrodus, 1967).

The gene, *GNLA*, encoding GS in *H. cylindrosporum*, has been cloned and characterized (Javelle *et al.*, 2003a). A single mRNA of 1.2kb was detected. Transfer of the fungus from an ammonium-containing to an N-free medium resulted in an increase of GS activity. However, when the mycelium was re-supplied with ammonium, GLNA transcripts remained unchanged or decreased only slowly, suggesting that GS in *H. cylindrosporum*, while being highly expressed, is not highly regulated.

The role of GDH should not be overlooked. In *H. crustuliniforme*, for example, assimilation of ammonium appears to be mainly via the GDH pathway (Quoreshi *et al.*, 1995). Two forms of this enzyme are recognized, one NAD (EC 1.4.1.2)- and the other NADP (EC 1.4.1.4)-dependent. The former has been ascribed a catabolic role, whereas the latter is considered to be involved in glutamate biosynthesis. Both have been found and characterized in the ECM fungus *L. laccata* (Dell *et al.*, 1989; Ahmad *et al.*, 1990; Brun *et al.*, 1992; Botton and Chalot, 1995; Garnier *et al.*, 1997). Its properties are similar to those reported for NADP-GDH enzymes of *Neurospora crassa* and yeasts (Stewart *et al.*, 1980). When GS is inhibited by methionine sulphoximine (MSX), glutamate, alanine and aspartate accumulate in mycelium of ECM fungi, confirming the presence and operation of the GDH pathway. These results suggest that ammonium assimilation, in these three fungi at least, is achieved by parallel action of GDH and GS-GOGAT (see also Chalot *et al.*, 1994a, 1994b). The need for examination of other fungi is highlighted by the observation that, in *Pisolithus tinctorius*, GS activity is low (Ahmad *et al.*, 1990) and that MSX blocked the synthesis of other amino acids suggesting the operation of the GS-GOGAT pathway. An NADH-dependent GOGAT has been detected in *L. bicolor* (Vezina *et al.*, 1989), but the instability of this enzyme renders characterization difficult and its status in ECM fungi remains uncertain.

The gene (*GDHA*) encoding NADP-GDH has been cloned and characterized from *L. bicolor* (Lorillou *et al.*, 1996), *H. cylindrosporum* (Javelle *et al.*, 2003a), and *Tuber borchii* (Vallorani *et al.*, 2002). Quantification of mRNA accumulation using a cDNA probe encoding the *L. bicolor* NADP-GDH confirmed that the growth of mycelia on nitrate and on N-free media resulted in an increased accumulation of transcripts encoding

NADP-GDH (Lorillou *et al.*, 1996). In the case of *H. cylindrosporum*, transfer of the fungus from 3 mM ammonium to N-free medium resulted in a 12-fold increase in the GDH transcript level, corresponding to a similar increase of enzyme activity. In contrast, feeding the mycelium with ammonium resulted in a rapid decrease of transcripts encoding GDH, which correlated with a decline in GDH-specific activity.

The studies outlined above provide an overview of the genes involved and aspects of the physiological events involved in assimilation of mineral N sources. However, they are heavily biased towards a very small number of ECM fungal species and analyses across a broader range of fungi, particularly those characteristic of distinctive soil types or ecosystems, are highly desirable.

Organic N sources

The ability of some ECM fungi to use organic N sources has been appreciated for many years (see Harley and Smith, 1983). Lundeberg (1970), in his cultural studies of a number of species and strains, identified several members of the genus *Suillus* that grew better on asparagine and glycine than they did on inorganic N. Again, there is clearly a good deal of variability between different species and even between strains of the same species. Laiho (1970) examined the N-source preferences of a number of strains of *Paxillus involutus*. All were able to use casein hydrolysate, peptone and a mixture of amino acids and all isolates grew well on both glutamate and arginine. Ability to grow on other amino acids as sole N source was low and variable. There was no case of an organic N source being very readily used by one strain and being totally useless to another.

To test the ability of ECM fungi to use organic N compounds in forest soil organic matter, Lundeberg (1970) prepared humus agar in which the concentrations of inorganic N compounds were reduced and the organic-N was labelled with ^{15}N. He allowed fungi to grow from an inoculum which straddled the interface between the N-free glucose agar and the humus agar. None of the ECM fungi absorbed organic N from the humus in significant quantities, although five other saprotrophic fungi which produced some or all of the hydrolytic enzymes, cellulase, pectinase, proteinase and laccase, were able to do so. These results agree with the findings of Mosca and Fontana (1975), on the use of protein-N by *Suillus luteus* and together such data have been taken to support the tentative conclusion that ECM fungi may not be very effective at acquiring organic N and must compete with other organisms for inorganic N mineralized by free-living members of the soil microflora.

Awareness of the possible importance of amino acids as primary sources of N for ECM fungi and for the plants which they colonize has been heightened by the recognition that these can constitute a significant pool, particularly in acid organic soils (Nemeth *et al.*, 1987; Abuarghub and Read, 1988a; Kielland, 1997), leading to re-examination of the role of ECM fungi in their mobilization. Some amino acids and amides, including glutamine, glutamate and alanine, which appear to occur in significant amounts in soil solution, are readily assimilated by ECM fungi in pure culture and, at equivalent concentrations of N and C, they can support yields as large as those obtained on ammonium as sole N source (Abuzindah and Read, 1988a; Finlay *et al.*, 1992; Keller, 1996; Anderson *et al.*, 1999) (Table 9.1).

Using *Paxillus involutus*, Chalot *et al.* (1994a, 1994b) demonstrated, by means of tracer and enzyme inhibition techniques, that glutamate, glutamine and alanine

Table 9.1 Yields of three ectomycorrhizal fungi, *Suillus bovinus*, *Amanita muscaria* and *Hebeloma crustuliniforme*, when grown with a range of mineral or amino N sources at a concentration of 60 mg N/l and at the same C:N ratio.

Nitrogen source	Wt of N source added (g)	Wt of glucose added (g)	Dry weight yields (mg) after 30 days		
			S. bovinus (mean ± SE)	A. muscaria (mean ± SE)	H. crustuliniforme (mean ± SE)
Mineral N.					
$Ca(NO_3)_2\ 4H_2O$	0.504	3.004	17.7 ± 1.5	4.7 ± 0.3	12.6 ± 1.2
$(NH_4)_2\ SO_4$	0.284	3.004	30.7 ± 1.2	26 ± 1.2	25.3 ± 1.4
Acidic amino acids					
L-Aspartic acid	0.572	2.372	31.3 ± 2.6	34.3 ± 2.0	23.0 ± 0.9
L-Glutamic acid	0.632	2.216	32.0 ± 1.0	36.3 ± 1.5	24.9 ± 1.3
Basic amino acids					
L-Arginine	0.224	2.764	33.6 ± 1.8	34.0 ± 2.5	15.6 ± 0.7
L-Lysine	0.392	2.528	17 ± 0.6	18.0 ± 0.1	5.9 ± 0.5
L-Histidine	0.300	2.688	5.0 ± 0.0	14.7 ± 1.2	4.2 ± 0.4
Neutral amino acids					
L-Alanine	0.380	2.528	31.6 ± 0.3	21.7 ± 1.2	20.4 ± 1.3
L-Asparagine	0.284	2.688	29 ± 1.5	23.3 ± 1.9	19.0 ± 0.7
L-Cysteine	0.520	2.528	7.7 ± 0.3	3.3 ± 0.8	6.0 ± 0.5
L-Cystine	0.516	2.528	6.0 ± 0.6	19.7 ± 0.7	5.6 ± 0.2
L-Glutamine	0.312	2.608	29.3 ± 1.4	36.3 ± 0.3	18.1 ± 0.7
L-Methionine	0.640	2.216	7.7 ± 0.3	2.3 ± 0.3	3.5 ± 0.2
Glycine	0.324	2.688	5.7 ± 0.3	22.7 ± 1.4	9.9 ± 0.4
L-Phenylalanine	0.708	1.588	11.3 ± 0.7	4.7 ± 0.7	4.3 ± 0.7
L-Hydroxy-L-proline	0.560	2.528	3.7 ± 0.3	4.7 ± 0.3	4.2 ± 0.5
L-Isoleucine	0.564	2.216	22.3 ± 2.3	7.3 ± 0.9	11.7 ± 0.3
L-Leucine	0.564	2.216	23.0 ± 3.2	7.3 ± 0.3	8.1 ± 0.4
L-Proline	0.492	2.216	4.0 ± 0.6	6.3 ± 0.9	4.4 ± 0.3
L-Serine	0.452	2.528	27.7 ± 0.9	18.7 ± 0.7	16.3 ± 1.2
L-Threonine	0.512	2.372	4.7 ± 0.3	6.7 ± 0.3	7.6 ± 0.3
L-Tryptophane	0.436	2.136	2.0 ± 0.0	2.7 ± 0.7	7 ± 0.1
L-Tyrosine	0.776	1.588	1.7 ± 0.3	5.3 ± 0.9	8.2 ± 1.0
L-Valine	0.300	2.220	17 ± 1.2	11.3 ± 0.9	18.9 ± 3.1
Minus N	–	3.004	7.3 ± 0.7	4.3 ± 0.3	6.2 ± 0.2

From Abuzinadah and Read (1988).

were absorbed intact and incorporated respectively by GS, GOGAT and alanine aminotransferase (AIAT) into the various assimilation pathways. Here the N sources would supplement the free amino acid pools, which represent important sinks for C in mycelia fed with mineral sources of N. It has been shown, for example, in *Cenococcum geophilum* and *Sphaerosporella brunnea* that 16–40% of C, fed as 1-^{13}C glucose to the mycelium, entered the amino acid pools (Martin and Canet, 1986; Martin *et al.*, 1988b). Exogenous supply of amino acids and amides would therefore be expected to supplement the organic C, as well as the N economy of ECM fungi.

In studies of assimilation of glutamine, glutamate and alanine carried out over a range of pHs, with and without the presence of ammonium, nitrate and glucose,

Chalot *et al.* (1995) showed that amino acid absorption had a distinctly acid pH optimum (Figure 9.2) and that neither ammonium, at concentrations from 0.05 to 0.5 mM, the low range (similar to those in soil), nor glucose, had an impact upon uptake. Over a period of 5–6 weeks, uptake of amino acids decreased by a factor of 4–10 as the mycelium aged, while the size of endogenous pools progressively increased. Extrapolation of information on pool turnover in pure cultures to symbiotic systems must be done with caution, because of the absence of a sink for assimilated N compounds which in ECM would be provided either by ongoing growth of the fungus in soil or by the associated plant.

It is widely accepted that amino acids are transported by proton symport mechanisms. Analysis of the energetics and specificity of a general amino acid transporter in *P. involutus* (Chalot *et al.*, 1996) showed that, in the case of glutamate, the concentration-dependent uptake failed to obey simple Michaelis Menten kinetics. Rather, there were two separate components of uptake, one of which was saturable and carrier mediated, the other a non-saturable diffusion-like process. The pH-dependence of amino acid uptake of this fungus was again demonstrated, it being optimal between pH 3.9 and 4.3 in the cases of glutamine and glutamate, and between 3.9 and 5.0 for alanine and aspartate. Both pH dependence and susceptibility of the uptake processes to inhibitors such as 2,4 dinitrophenol (DNP) are consistent with a proton symport mechanism for amino acid uptake by *P. involutus*. Competition

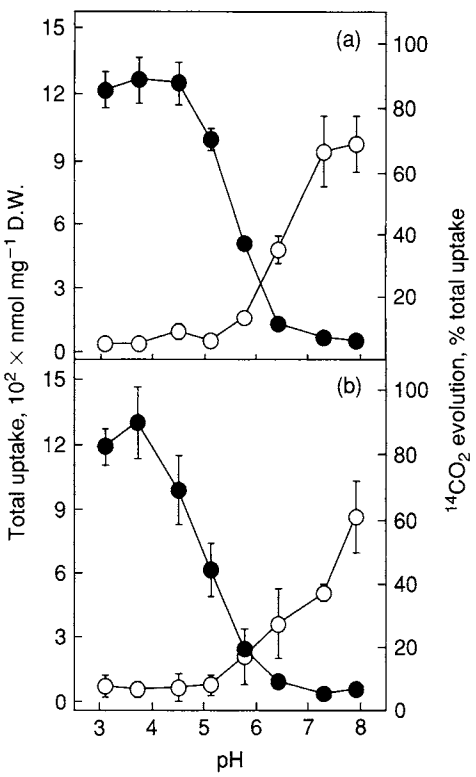


Figure 9.2 Effect of external pH on the uptake (●) and respiration (○) of L-glutamate and L-glutamine by the ectomycorrhizal fungus *Paxillus involutus*. Data are means of three replicate determinations ± standard errors of the means. From Chalot *et al.* (1995), with permission.

studies in this fungus (Chalot *et al.*, 2002, 2006) suggest a broad substrate recognition that is consistent with the role of a general amino acid permease.

A gene encoding an amino acid importer (*AmAAP1*) of *Amanita muscaria* has been isolated and the activities of its transcripts characterized by heterologous expression in yeast (Nehls *et al.*, 1999). This study confirmed the protein to be a high-affinity, general amino acid permease with a K_m of 22 μm for histidine and up to 100 μm for proline. A low constitutive expression of the transporter was detected in the presence of amino acids. By contrast, under conditions of N starvation, or in the presence of nitrate or phenylalanine, neither of which is used as an N source by *A. muscaria*, expression of the gene was considerably enhanced. It was concluded that in *A. muscaria*, as in yeast and *Aspergillus nidulans*, gene expression of amino acid transporters is regulated at the transcriptional level by N repression (Nehls *et al.*, 1999). *AmAAP1* was shown to have a higher affinity for basic than for acidic or neutral amino acids, which may reflect a preponderance of basic amino compounds in acidic forest soils. Nehls *et al.* (1999) raise the possibility that, in addition to having a role in amino acid uptake for nutrition, the enhancement of expression under conditions of N starvation may prevent amino acid loss by leakage from hyphae when suitable N sources are lacking. In a complementary study, Wipf *et al.* (2002) isolated an amino acid transporter (*HcBap1*) from *H. cylindrosporum* by functional complementation of a transporter-deficient yeast strain.

Whereas some of the amino acids present in the soil solution are undoubtedly derived from the free pools of these molecules in living plant and microbial tissues, others are likely to be a product of the cleavage of polymeric peptides and proteins associated by decomposer organisms in soil. Since the polymers constitute the bulk of the N in many ECM forest soils (see Chapter 15), it is relevant to determine whether they too might be accessible to ECM fungi. Abuzinadah and Read (1986a) showed that *Suillus bovinus* (Figure 9.3b), *Rhizopogon roseolus* and *Pisolithus tinctorius* readily used a series of alanine peptides of increasing chain length from the di- to the penta-peptide. Hexa-alanine was also used, but more slowly. *Laccaria laccata*, in contrast, made poor growth on all these substrates (Figure 9.3a).

In early experiments, Chalot and Brun (1998) reported that uptake of ^{14}C-labelled peptides was slower than that of amino acids. Later, Benjdia *et al.* (2006) demonstrated that *H. cylindrosporum* was capable of taking up di- and tripeptides and of growing on them as sole N sources. Using an *H. cylindrosporum* cDNA library, two peptide transporters (*HcPTR2A* and *B*) were isolated by means of yeast functional complementation and these were shown to mediate dipeptide uptake. *HcPTR2A* was involved in high-efficiency peptide uptake under conditions of limited N availability, whereas *HcPTR2B* was expressed constitutively.

The capacity of ECM fungi to use proteins as N sources has been known for some time (Melin, 1925; Lundeberg, 1970), but the extent of fungal proteolytic capability was not fully appreciated. Using soluble proteins of animal and plant origin, Abuzinadah and Read (1986a, 1986b) screened eight ECM fungi over a wide range of pH conditions for the ability to exploit these polymers as sole sources of N. On both bovine serum albumin (BSA, MW 67000, N content 16%) and gliadin (MW approx. 30000, N content 14%) some ECM fungi produced yields as large or even larger than those obtained with ammonium as sole N source. These, including *Amanita muscaria, Cenococcum geophilum, Paxillus involutus, R. roseolus, S. bovinus* and *H. crustuliniforme*, were referred to as 'protein fungi'. In contrast, *L. laccata* and

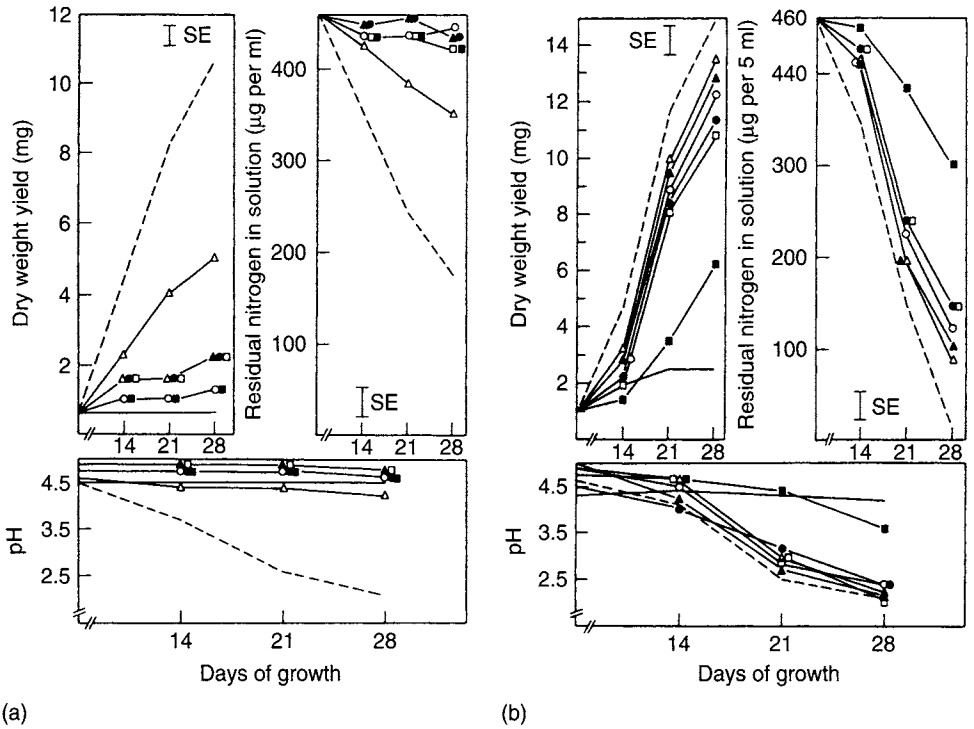


Figure 9.3 Dry weight of mycelium, residual N and pH of the culture medium (a) of *Laccaria laccata* and (b) *Suillus bovinus* grown in liquid medium containing ----- ammonium; Δ alanine; a range of alanine peptides of chain length 2–6 (▲ A2; ○ A3; ● A4; □ A5; ■ A6) or —, lacking N. Data are means of three replicates ± standard errors of the means. From Abuzinadah and Read (1986a), with permission.

Lactarius rufus, the so-called 'non-protein' fungi, produced only small yields on these organic N substrates. *P. tinctorius* was in an intermediate category. Subsequent examination of *Pisolithus* (Anderson *et al.*, 1999) showed a generally low level of ability to use BSA, but also considerable inter- and intraspecific variation.

El-Badaoui and Botton (1989) isolated a protein-rich fraction from forest litter and observed that it induced greater proteolytic activity in *A. rubescens*, *C. geophilum* and *H. crustuliniforme* than did BSA or gelatin. There is obviously variation in the accessibility of commercial proteins for fungal growth. For example, Hutchison (1990) screened a very large number of fungi for their ability to breakdown gelatin to its constituent amino acids but failed to detect activity even in species such as *H. crustuliniforme* and *P. involutus* which are known to be able to hydrolyse BSA or gliadin. The extracellular acid proteinase of *H. crustuliniforme* has been purified and characterized (Zhu *et al.*, 1990). The enzyme was most stable and had greatest activity over the pH range 2–5. Protein and some individual amino acids, notably glycine (Table 9.2), induced activity when they were supplied as sole N sources. Enzyme production was not affected by the addition of ammonium at 3.2 mM, but was repressed at higher concentrations. Production required the presence of a simple C source and was not repressed by glucose between 0.5 and 2.0% (Zhu *et al.*, 1994). Consequently, it appears that, as in the case of the ERM fungus *Rhizoscyphus ericae* (Leake and

Table 9.2 Effect of various nitrogen sources on growth and proteinase production of *H. crustuliniforme* after 20 days of growth[a].

Nitrogen sources[b]	Conc.	Dry weight (mg/plate)	Proteinase activity (units/mg dry wt)	(% of control)	Final pH
Ammonium	3.2 mM	21.3 ± 1.5	32.4 ± 2.1	Control	2.8
Asparagine	10 mM	36.1 ± 3.4	16.7 ± 1.2	52	5.4
Glycine	10 mM	10.4 ± 1.6	74.7 ± 3.4	230	5.3
Glutamine	10 mM	89.2 ± 5.6	8.6 ± 0.9	27	6.5
Casein hydrolysate	0.04%	37.4 ± 2.7	9.6 ± 2.1	30	6.4
Casein	0.04%	36.1 ± 3.6	18.4 ± 2.7	57	4.9
Gelatin	0.04%	44.7 ± 3.2	60.1 ± 3.2	185	4.8
Gliadin	0.04%	25.7 ± 2.1	52.9 ± 4.6	163	4.1
BSA	0.04%	28.6 ± 2.4	70.8 ± 5.2	219	4.5

From Zhu *et al.* 1994. [a]Data are presented as mean ± standard deviation of five replicates. [b]Nitrogen sources were added to the basal medium containing 1% glucose.

Read, 1991; see Chapter 11), the acid proteinase of *H. crustuliniforme* is largely regulated by induction. It may be repressed by some forms of N, but catabolite repression in general does not seem to play an important part. The observation that amino acids supplied singly can act as inducers is important, in view of current recognition that soils of the kinds in which ECM roots grow can have sizeable pools of these compounds (Abuarghub and Read, 1988a; Kielland *et al.*, 1994).

Nehls *et al.* (2001c) isolated and characterized two aspartic proteases which are released in a pH-dependent manner by mycelia of *Amanita muscaria*. AmProt1 with a molecular mass of approx. 45 kDa is mainly produced up to pH 5.4, with an optimum around pH 3.0, whereas the excretion of AmProt2, which had a mass of 90 kDa, was only detectable at pHs between 5.4 and 6.3 and had a somewhat higher pH optimum for activity. One cDNA clone presumed to encode AmProt1 was identified and also shown to be expressed in a pH-dependent manner. C- and N-limitation significantly enhanced AmProt1 expression. However, whereas N starvation alone increased expression of the gene by three to fourfold, the absence of a C source increased transcript levels by a factor of 12 independently of the presence of N in the bathing medium. Thus, the endogenous C levels in ECM hyphae can be expected to exert strong regulatory controls on levels of proteolytic activity.

H. crustuliniforme, when grown on protein as sole N source shows a prolonged lag phase, followed by a phase of more rapid growth during which proteolysis results in accumulation of amino acids in the medium (Read *et al.*, 1989) (Figure 9.4). These are subsequently assimilated, but only after the protein concentration has been markedly reduced. At no stage during growth is there evidence of ammonium release. Indeed, ammonium ions are detectable in the medium only when the amino compounds have themselves been virtually exhausted. By this stage, C-starvation of the fungus is likely to have led to deamination of the residual compounds and release and utilization of the C-skeletons. Such starvation and the release of ammonium associated with it, may be both artefacts of the pure culture environment and unrepresentative of the symbiotic condition in which C supply from the plant is assured. Furthermore, the failure of ammonium to appear in the medium at earlier stages of growth could

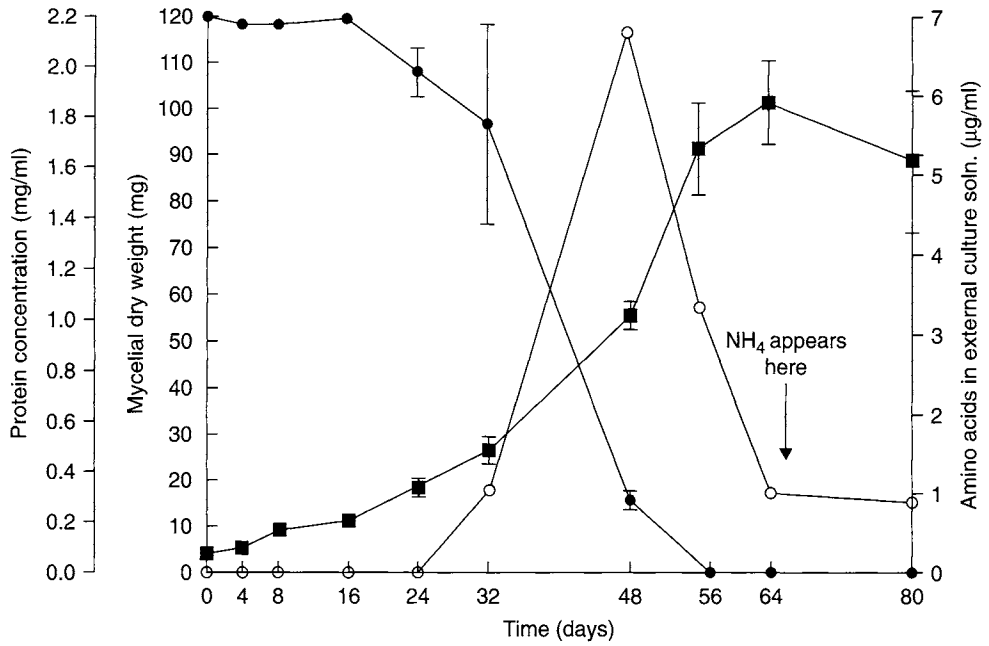


Figure 9.4 The relationship between protein utilization, mycelial biomass production and amino acid release when *Hebeloma crustuliniforme* is grown in liquid culture with protein as the sole N source. Note that ammonium does not appear in the medium until all the protein has been used and the fungus therefore starved of C. ● protein concentration; ○ amino acid concentration; ■ mycelial dry weight. Data are standard errors of means. From Read *et al.* (1989), with permission.

reflect the relative rates of deamination of protein (if that occurs) and uptake of ammonium which would be likely to be rapid in actively growing mycelium.

The availability of proteins in soil depends on their structure and solubility and on their interactions with other soil components. Thus, the relatively insoluble gelatin is less available in culture than the soluble BSA but, in soil, all proteins might be rendered unavailable by tanning reactions with phenolic compounds, by ionic reactions with soil organic matter and clays, or by physical occlusion in the soil pores. The success of ECM fungi in obtaining these less-available resources might very well be increased, compared with saprotrophic fungi because, in symbiosis, they are not dependent on soil organic C. For this reason, if for no other, it is clearly essential to extend studies of the abilities of the fungi using intact symbiotic systems in which the normal pathways of organic C supply would be operating.

Use of N by mycorrhizal roots and intact plants

Inorganic N sources

In a comparative analysis of the abilities of ECM and non-mycorrhizal roots of *Picea abies* to take up nitrate and ammonium, Eltrop and Marschner (1996) reported that neither the mycorrhizal status of the plants nor the form of N supplied greatly influenced

Table 9.3 Kinetic uptake parameters (substrate affinity K_m in μM, and maximal uptake rate V_{max} in μmol/g f.wt/h, after Lineweaver-Burk) for ammonium and nitrate in intact root systems of 22-week-old non-mycorrhizal and mycorrhizal Norway spruce seedlings.

	Ammonium		Nitrate	
	K_m	V_{max}	K_m	V_{max}
Supply of N as NH_4NO_3				
Non-mycorrhizal	227	0.38	214	0.28
Laccaria laccata	229	0.55	207	0.33
Paxillus involutus	222	0.47	228	0.32
Pisolithus tinctorius	236	0.53	367	0.51
Supply of N as $(NH_4)_2SO_4$ or KNO_3				
Non-mycorrhizal	567	0.96	98	0.46
Pisolithus tinctorius	334	1.24	263	0.97

Nitrogen was supplied either as NH_4NO_3 or as $(NH_4)_2SO_4$ or KNO_3 at a total concentration of 800 μm. Data from Eltrop and Marschner (1996).

Table 9.4 Kinetic parameters for methylammonium and amino acid uptake by mycorrhizal beech roots calculated by non-linear regression analysis in a substrate concentration range of 0.001–0.25 mol/m.

Fungus	Compound	V_{max}	K_m
Lactarius subdulcis	Methylammonium	48.5 ± 6.2	220 ± 62
	Glutamine	12.4 ± 0.9	79 ± 13
	Glycine	22.8 ± 4.2	197 ± 67
	Glutamic acid	10.1 ± 1.3	115 ± 33
Russula ochroleuca	Methylammonium	27.9 ± 7.2	555 ± 191
	Glutamine	1.4 ± 0.1	86 ± 20
	Glycine	3.1 ± 0.3	233 ± 35
Xerocomus chrysenteron	Methylammonium	40.8 ± 8.0	441 ± 122
	Glutamine	16.5 ± 1.5	80 ± 18
	Glycine	18.7 ± 2.5	84 ± 26

Values for V_{max} (μmol/g DW/h) and K_m (mmol/m^3) (±SE of the fitted parameters) were calculated by non-linear curve fitting of the experimental data to the Michaelis-Menten equation [$v = (V_{max} \times [S])/(K_m + [S])$] in a substrate concentration range of 0.001–0.25 mol/m^3. Sampling site: Hathersage, Derbyshire, UK. From Wallenda and Read (1999).

the kinetic properties of the uptake process (Table 9.3). K_mS for uptake of nitrate were slightly lower than those for ammonium, suggesting a higher affinity for the former. Caution must, however, be exercised when generalizing from such studies because it was subsequently shown that uptake rates of methyl ammonium, as an analogue for ammonium, are strongly fungus-dependent roots colonized by *Lactarius subdulcis* having higher affinities for the analogue than those supporting *Russula ochroleuca* (Wallenda and Read, 1999) (Table 9.4). This variation might explain some of the large variability seen in K_m for methyl ammonium uptake observed by Kielland (1994) in field collected ECM roots of *Salix* and *Betula* (Table 9.5).

Table 9.5 Kinetic parameters for methylamine (ammonium) uptake by excised roots of major plant species from four Arctic tundra communities.

Community	Species	Mycorrhizal type	Half saturation constant, K_m (μmol/l)	Maximum uptake capacity, V_{max} (μmol/g/h)
DH	*Betula nana*	ECM	67	5.6
WM	*Carex aquatilis*	NM	153	17.8
	Eriophorum angustifolium	NM	935	17.8
TT	*Betula nana*	ECM	3821	142.8
	Carex bigelowii	NM	78	5.4
	Eriophorum vaginatum	NM	242	13.7
	Ledum palustre	ERM	256	7.6
	Salix pulchra	ECM	1197	75.9
ST	*Betula nana*	ECM	663	31.7
	Salix pulchra	ECM	6717	272.8

Kinetic constants were calculated from four replicate measurements at each of four solution concentrations. Abbreviations: DH = dry heath, ECM = ectomycorrhizal, ERM = ericoid, NM = non-mycorrhizal, TT = tussock tundra, ST = shrub tundra and WM = wet meadow. From Kielland (1994).

The fate of ^{15}N from labelled nitrate and ammonium fed to external mycelium attached to intact ECM plants was studied by Finlay *et al.* (1988, 1989) and by Ek *et al.* (1994a). Glutamine was identified as a major sink for absorbed N, with alanine, arginine and aspartate-asparagine also important (Finlay *et al.*, 1992; Martin and Botton, 1993; Botton and Chalot, 1995). The contribution of arginine to the pool of amino acids and amides differs with fungal species and the suggestion has been made that this may relate to variations in accumulation in vacuoles, where arginine might play a role in stabilizing polyphosphate (polyP) (Finlay *et al.*, 1992; see Chapter 10). The data obtained with ^{15}N on intact ECM plants are consistent with earlier findings using ^{14}C with excised roots, confirming that glutamine is most likely to be the major form in which N is translocated in the mycelium and transferred across the symbiotic interface to the plant (Harley, 1964; Carrodus, 1967; Reid and Lewis, in Lewis, 1976). There is no doubt that transfer occurs very rapidly in the intact systems (Finlay *et al.*, 1988, 1989). ^{15}N-labelling experiments have shown that ammonium is preferentially absorbed from ammonium nitrate by *Paxillus involutus* in association with *Betula pendula* and also that ammonium inhibited $^{15}NO_3$ assimilation in the external hyphae (Ek *et al.*, 1994a). Again, the ammonium was assimilated into glutamine at the uptake site, providing evidence for the importance of this amide in translocation.

France and Reid (1983) provided a conceptual model of the mechanisms thought to be important for assimilation of ammonium in ECM roots, in which the pathways involving GS, GOGAT and GDH were all present. Subsequent studies (Martin and Botton, 1993; Botton and Chalot, 1995) have revealed a diversity of pathways in which the plant exercises considerable influence over the activities of the fungi in the symbiotic condition, as well as important differences between plant–fungus combinations (Figure 9.5). Whereas the GDH-GS pathway appears to predominate in the fungi, ammonium assimilation in higher plants is known to occur primarily via

GS-GOGAT (Robinson *et al.*, 1991; Oaks, 1994; Miflin and Lea, 1976). If fungal and higher plant cells have distinctive pathways of inorganic N assimilation, it is important to determine how these are controlled in the ECM condition. In some cases, the fungal enzymes are downregulated in proximity to plant tissues, a feature that gave rise to the concept of metabolic zonation (Martin *et al.*, 1992) while, in others, activity is relatively unaffected. Thus, in *Picea* mycorrhizas formed by *Hebeloma*, NADP-GDH activity was highest in the extraradical hyphae and mantle, but reduced in the Hartig net (Dell *et al.*, 1989). In line with this, the quantity of GDH polypeptide, revealed by immunogold labelling, decreased progressively from the peripheral cells of the mantle to the Hartig net (Chalot *et al.*, 1990a). In *Fagus* mycorrhizas, on the other hand, both the activity (Dell *et al.*, 1989) and the amount (Chalot *et al.*, 1990a) of GDH were strongly suppressed throughout the mantle whether it was formed by *Hebeloma* or by *Cenococcum geophilum* or *P. involutus*.

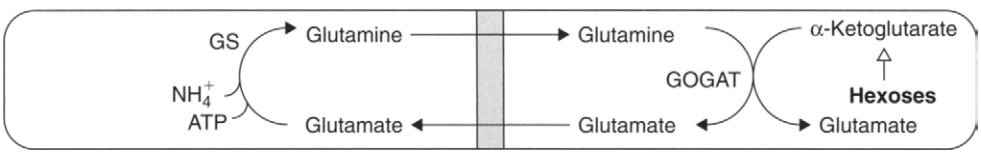
A similar picture was revealed in *Fagus* roots with *Lactarius*-type ECM with successive incorporation of $^{15}NH_4$ into the amido-N of glutamine, glutamate and alanine, consistent with GS activity (Martin *et al.*, 1986). The importance of this pathway was confirmed by incubation of roots in MSX which inhibited ^{15}N-incorporation into glutamine and glutamate by 90%. Supporting evidence for the role of GS-GOGAT was obtained by use of the GOGAT inhibitor azaserine which completely blocked glutamate synthesis from ammonium. Such observations are suggestive of a complete downregulation of fungal GDH in some ECM types, although there remains the possibility of expression of some activity in the mycelium as it grows away from the root. In addition, N sources (ammonium versus nitrate) and concentration (N-rich versus N-starved) regulate NADP-GDH biosynthesis and activity through the alteration of GDH mRNA accumulation in *L. laccata* S238 (Lorillou *et al.*, 1996), suggesting the potential for regulation by N availability in soil.

Further evidence for the role of ammonium concentration in controlling pathways of ammonium assimilation in the fungi (albeit from studies in pure culture) comes from the use of NMR spectroscopy to monitor ^{15}N-labelling, after feeding *L. biolor* with $^{15}NH_4$ (Martin *et al.*, 1994). Rapidly growing mycelium assimilated $^{15}NH_4$ into glutamine via GS, which was probably the main route. However, when this pathway was inhibited by MSX, GDH activity became apparent. In stationary-phase growth with low concentrations of ammonium in the medium, both pathways operated.

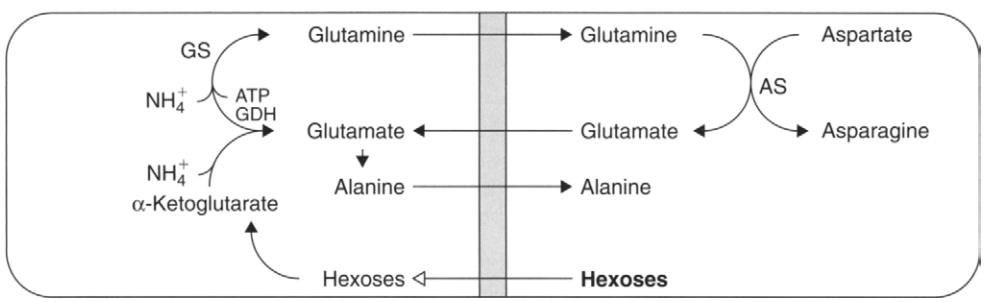
The pathways of ammonium assimilation and the distribution of the enzymes involved have important implications for the mechanisms of transport of N across the fungus–root interface. Martin and Botton (1993) recognized three basic patterns of N incorporation. In *Fagus*, GS is localized in the fungal mantle and GOGAT in the root. In *Picea*, GDH and GS occur in extraradical mycelium and mantle respectively, with asparagine synthetase (AS) in the root. A third pattern (Figure 9.5) is representative of ECM formed by *Pisolithus tinctorius*, in which the GS-GOGAT pathway appears to operate in this fungus (Figure 9.5). It should be noted that, although Figure 9.5 shows transfer of hexoses from plant to fungus, this is an oversimplification because (as shown in Chapter 8) transfer of organic C probably involves sucrose efflux to the apoplast and its hydrolysis to hexoses before uptake by the fungus.

The question of uptake and transport of solutes can best be addressed in systems in which the sources of nutrient supply (the soil) and the ultimate sinks (the transpiring shoots) are intact. Enhancement of ammonium uptake was demonstrated when intact seedlings of *Tsuga heterophylla*, *Picea sitchensis* and *Pseudotsuga menziesii* were

colonized by *H. crustuliniforme* compared with non-mycorrhizal seedlings, over a pH range of 3–7 (Rygiewitz *et al.*, 1984). Significantly greater rates of uptake were maintained in all three species when the plants were mycorrhizal. However, absolute amounts absorbed were always lower in both ECM and non-mycorrhizal plants under acidic conditions. Finlay *et al.* (1988) fed $^{15}NH_4$ to the distal parts of mycelial systems of *Rhizopogon roseolus*, *Suillus bovinus*, *Paxillus involutus* and *Pisolithus tinctorius* growing across peat from colonized roots of *Pinus sylvestris*. Measurements of the distribution of ^{15}N after 72 hours showed high labelling in glutamate-glutamine, aspartate-asparagine and alanine in all plant–fungus combinations, except that involving *P. involutus* which showed no labelling in aspartate-asparagine. Within this time period, 5–50% of the amino acids in the shoot were also labelled, indicating the very rapid operation of the translocation and transfer processes. Within the plant, despite large differences in the size of the different amino acid pools, the levels of ^{15}N-enrichment were similar, indicating that equilibrium between the pools, dependent upon activities of amino transferases, was rapidly achieved.



(a)



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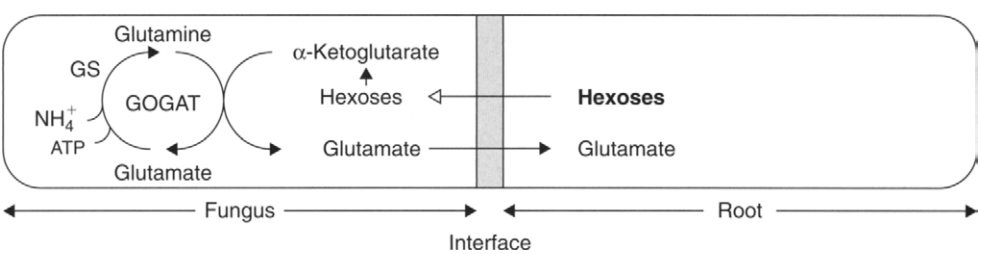

(c)

Figure 9.5 Possible localization of enzymes of ammonium assimilation in different types of ectomycorrhizas. (a) *Fagus*: glutamine synthetase (GS) in the fungus and glutamate synthase (GOGAT) in the root. (b) *Picea*: NADP-glutamate dehydrogenase (GDH) and GS in the fungus and asparagine synthetase (AS) in the root. (c) *Pisolithus* mycorrhizas: GS and GOGAT in the fungus. Modified from Martin and Botton (1993).

An alternative to the view that ammonium assimilation takes place primarily by GS-dependent glutamine accumulation has been proposed by Chalot *et al.* (2006). This suggests (Figure 9.6) that N taken up as ammonium may be translocated and transferred across the ECM interface, either as ammonium or uncharged ammonia. Evidence in support of this possibility comes from the description of a root-specific high-affinity ammonium importer (*PttAMT1.2*) in aspen (*Populus tremula*), the expression of which was greatly increased in an N-independent manner upon ECM formation (Selle *et al.*, 2005). Strong downregulation of genes coding for GS in fungal cells in close contact to the root (Wright *et al.*, 2005) and decreases of the activity of enzymes involved in N assimilation during ECM colonization (Blaudez *et al.*, 1998) would contribute to the maintenance of ammonium concentrations at the fungus–plant interface that are necessary to achieve transfer.

The nature of any possible ammonium transfer mechanisms remains a matter of conjecture (Selle *et al.*, 2005; Chalot *et al.*, 2006). Diffusion of ammonia across the plasma membrane into the interfacial apoplast of the Hartig net is a possibility. If this occurred, the low ambient pH of the apoplast (Nenninger and Heyser, 1998) would lead to protonation of ammonia and prevention of its return by diffusion. The N-dependent repression of the ammonium importer gene of the fungal partner observed by Javelle *et al.* (2003a) could also be expected to contribute to inhibition of back-flow. Ammonium efflux proteins of the kind reported in yeast (Guaragnella and Butow, 2003) could contribute to ammonium export at the fungus–plant interface. Tarkka *et al.* (2006) report that genes encoding proteins homologous to those in yeast are expressed in *Amanita muscaria*. The possible involvement of non-specific channels, such as aquaporins, and of voltage dependent cation channels in ammonium import by ECM plants have also been suggested (Chalot *et al.*, 2006).

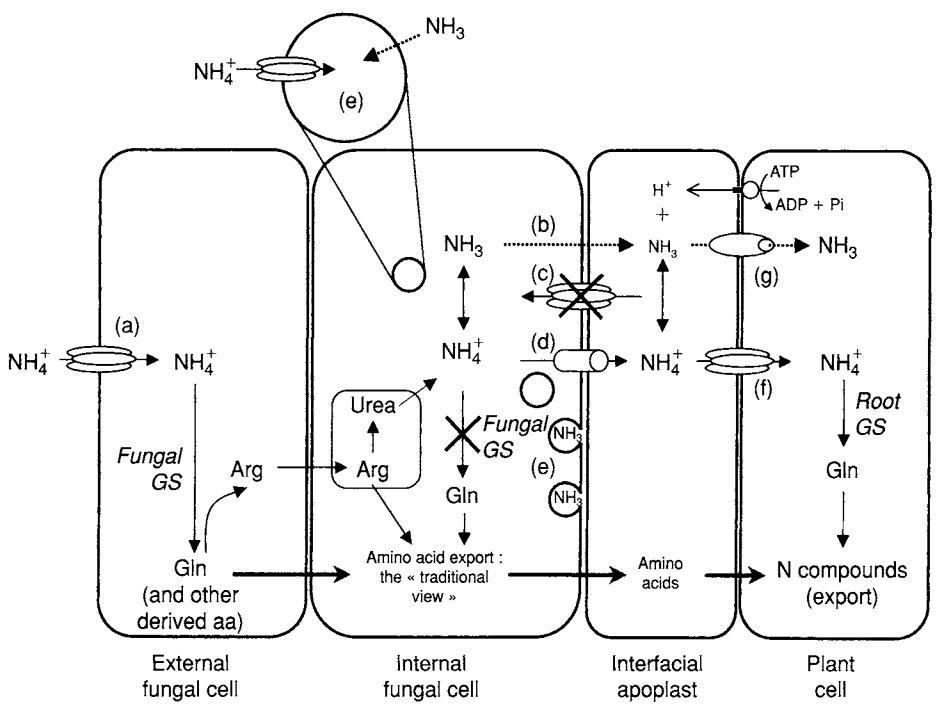

Figure 9.6 (Caption on p. 338)

The possibility that ammonium may be at least one of the forms of N transferred across the ECM interface has been raised at the same time as a similar scenario has been revealed for AM symbioses (see Chapter 5). For both associations it now needs to be appreciated that transfer of ammonium or ammonia will incur potential problems relating to pH regulation and charge balance (Raven and Smith, 1976). Sorting out how the plants meet these challenges will be an important topic in future research.

Studies of the impact of ECM colonization upon nitrate assimilation and growth of the plant made clear the differences between patterns seen in the axenic and symbiotic systems. Scheromm *et al.* (1990a), using the isolate of *H. crustuliniforme* shown in pure culture to assimilate nitrate preferentially, could find no direct effect of the fungus upon either uptake or reduction of nitrate by mycorrhizal *Pinus pinaster*. Using the same fungus, Rygiewitz *et al.* (1984) observed no differences in nitrate uptake between colonized and uncolonized plants of *Picea sitchensis* or *Tsuga heterophylla*. Increases in uptake of nitrate were, however, observed in colonized *Pseudotsuga* seedlings. pH was the major factor determining uptake of nitrate in both categories of plant, it being increased as pH was increased.

Figure 9.6 Possible ammonium transfer mechanisms (a–g) across different mycorrhizal interfaces as identified by Chalot *et al.* (2006). (a) After soil-derived ammonium is taken up by plasma membrane-based ammonium transporters (Amts) of the external hyphae, specific septal pores will ensure symplastic continuity and cell-to-cell transport of ammonia. N transfer between fungal and root cells will occur at the interfacial apoplast, either as organic N compounds according to the 'traditional view' (bottom horizontal arrows), or as ammonia. Transport mechanisms for organic nitrogen, which have yet to be revealed, would be of crucial importance if N is taken up by the fungus in organic form. (b) At ammonium concentrations in the millimolar range measured in the fungal cells, ammonia concentration in the cytosol can be far higher than in the apoplast, thus generating a favourable gradient for passive diffusion of ammonia. Such a model requires apoplastic concentrations to be kept low, potentially by an ammonia acid-trapping mechanism provided by the low pH thought to occur at the mycorrhizal interface and owing to the increased activity of H^+-ATPase along the plant membrane flanking the interface. An excretion mechanism based predominantly on ammonia diffusion is questionable because of the lack of overall control of the process. A large array of membrane proteins could fulfil the function of transferring ammonia from the fungal cytosol to the interfacial apoplast. (c) It is anticipated that the number of Amts would be decreased at the plasma membrane in ECM mantle hyphae compared with those in the extraradical hyphae. (d) Transcriptomic studies in various ECM associations have identified fungal efflux systems that could be promising candidates for the release of ammonia at the fungal membrane. (e) Alternatively, diffusion of ammonia or active transport of ammonium via an Amt-mediated system into acidified-vesicles would ensure compartmentation of excess ammonia. The ammonia-loaded vesicles could then move via microtubules to the symbiotic membrane where vesicles would fuse with the plasma membrane and release ammonia into the interfacial apoplast. (f) The further transfer of ammonia from the apoplast to the plant host cytoplasm might involve plant Amts. (g) Non-specific channels such as aqua-ammoniaporins or voltage-dependent cation systems might also contribute to ammonia transport from the interfacial apoplast to the plant cell cytoplasm. Abbreviations: Arg, arginine; Gln, glutamine; GS, glutamine synthetase. From Chalot *et al.* (2006).

Table 9.6 Levels of ^{15}N enrichment (atom % excess) in free amino acids in ectomycorrhizal systems of *Fagus sylvatica* infected with the fungus *Paxillus involutus* and supplied with ^{15}N-labelled ammonium or nitrate.

N source	Mycelium		Mycorrhizal tips		Roots		Shoots	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Amino acid								
Ala	30.8	57.9	25.9	38.6	6.4	13.0	0.0	0.0
Gly	11.5	24.7	4.7	7.9	0.0	1.9	0.0	0.4
Thr	2.5	8.0	1.1	1.6	0.8	2.4	0.0	0.0
Ser	8.6	26.6	3.1	41.3	0.9	6.5	0.1	2.0
Leu	0.0	6.7	0.0	3.7	2.7	7.1	0.0	0.5
Ileu	3.5	5.1	0.9	6.6	5.5	7.8	0.0	0.0
Gaba	30.8	61.5	22.0	29.8	5.1	16.2	0.0	1.0
Pip	0.0	0.0	11.9	1.3	0.0	0.3	0.0	0.0
Asx	30.0	53.9	14.0	17.7	4.6	16.9	1.4	1.3
Glx	37.7	68.5	25.3	51.1	6.4	21.0	0.0	2.1
Lys	0.9	5.8	1.2	3.5	1.3	3.1	0.0	0.0
Tyr	9.9	10.7	2.6	5.3	1.0	13.2	0.0	9.5
Arg	1.2	0.0	6.8	11.1	0.4	4.1	0.0	0.0
No. 1	38.1	69.6	28.5	56.1	6.6	23.3	0.0	1.4
Mean	14.7	28.5	10.6	19.7	2.9	9.8	0.1	2.1

From Finlay *et al.* (1989). Values represent means of duplicate growth chambers. No. 1 represents an unidentified amino acid. Pip = pipecolinic acid.

Table 9.7 Levels of ^{15}N enrichment (atom % excess) in protein-incorporated amino acids in ectomycorrhizal systems of *Fagus sylvatica* infected with the fungus *Paxillus involutus* and supplied with ^{15}N-labelled ammonium or nitrate.

N source	Mycelium		Mycorrhizal tips		Roots		Shoots	
	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+	NO_3^-	NH_4^+
Amino acid								
Ala	1.1	4.7	3.8	6.8	1.6	4.1	0.3	0.3
Gly	0.2	1.6	3.1	3.4	1.5	3.0	0.2	0.0
Val	1.2	2.8	2.6	3.4	1.5	2.4	0.0	0.2
Ser	1.3	1.9	2.9	2.9	1.6	2.3	0.2	0.0
Leu	1.8	4.6	4.4	5.3	1.8	3.2	0.1	0.0
Ileu	1.1	3.6	3.7	4.8	1.7	3.1	0.0	0.0
Pro	0.6	3.3	1.9	2.8	1.2	1.9	0.0	0.0
Asx	2.1	5.4	5.0	6.2	2.0	4.5	0.4	0.0
Phe	1.0	2.8	3.2	3.7	2.3	3.5	0.1	0.0
Glx	3.5	9.3	6.0	8.3	2.2	4.4	0.2	0.0
Lys	1.3	1.1	1.4	1.5	0.7	1.1	0.3	0.0
Tyr	1.0	0.1	0.8	1.5	0.5	1.6	0.5	0.0
Arg	3.2	1.4	2.9	1.3	1.2	3.4	1.3	1.4
Mean	1.6	2.7	2.8	3.4	1.4	2.6	0.3	0.2

From Finlay *et al.* (1989). Values represent means of duplicate growth chambers.

Finlay *et al.* (1989) fed $^{15}NO_3$ to the extraradical mycelium of *Paxillus involutus* associated with *Fagus sylvatica* and compared the patterns of uptake, assimilation and transport of the label with those obtained in systems fed with $^{15}NH_4$. ^{15}N was taken up from both sources, incorporated into a range of free amino acids and transported to the shoots (Table 9.6). However, the amounts of enrichment of most free and protein-bound amino acids were usually greater in the systems fed with $^{15}NH_4$, than in those fed with nitrate. N assimilated from nitrate was only 62% of that obtained from ammonium (Table 9.7). Interesting though these results are, the immense genetic variability, even within races of *P. involutus* itself (Laiho, 1970) with respect to nitrate utilization must be borne in mind. The possibility remains that, where roots of trees such as *Fagus* grow in nitrifying environments, selection may favour colonization by fungi which preferentially use nitrate.

Organic N sources

Since Melin and Nilsson (1953a) demonstrated uptake and transfer of ^{15}N-glutamate by *Suillus granulatus* in ECM association with *Pinus*, there have been surprisingly few studies of amino acid utilization by intact ECM plants. Alexander (1983) fed aspartic acid and serine at concentrations of 0.5 mM to ECM and non-mycorrhizal *Picea sitchensis*. Whereas both N sources depressed growth of non-mycorrhizal seedlings, the inhibitory effect of aspartic acid did not occur in ECM plants and that of serine was reduced. Clearly, at high concentrations, toxicity of amino acids can be a problem for both fungus and plant. Accumulation of alanine, arginine and aspartic acid in ECM roots of *Pseudotsuga menziesii* and *Tsuga heterophylla* has been demonstrated in short-term uptake studies using colonized and uncolonized plants (Sangwanit and Bledsoe, 1987). Wallenda and Read (1999) carried out a comparative analysis of the uptake kinetics of the amino acids glutamine and glycine by a range of ECM types excised from pine, spruce and beech. All types took up amino acids via high-affinity transport systems. The kinetic parameters (Table 9.8) indicated that ECM roots have similar or even higher affinities (lower K_m values) for glutamine and glycine than for methylammonium. This confirms the potential of these organic forms to contribute significantly to total N uptake by ECM plants. The data further demonstrated that, as in the case of ammonium (see above), the considerable differences in the kinetic properties of amino acid uptake seen within a single species of plant are determined by the fungal partner.

It has been demonstrated that ECM colonization of birch (*B. pendula*) by *P. involutus* led to major changes in the metabolic fate of exogenously supplied amino acids (Blaudez *et al.*, 2001). The uptake of ^{14}C glutamate was increased by up to eight times, especially in the early stages of ECM formation. In addition, it was shown that glutamine was the major ^{14}C sink in ECM roots. In contrast, citrulline and insol-uble compounds were the major compounds to be labelled in non-mycorrhizal roots.

When grown with alanine or its peptides as sole N sources over a longer period of time, *Betula* showed striking responses to ECM colonization (Abuzinadah and Read, 1989a). In the absence of colonization, birch seedlings appeared to have no ability to use alanine and were N deficient. In contrast, when colonized by *Hebeloma crustuliniforme*, *Amanita muscaria* or *P. involutus*, the plants grew vigorously and their tissues contained N concentrations of the kind seen in healthy plants. When the plants were supplied with peptides of alanine of chain lengths from two to six units, again,

Table 9.8 Kinetic parameters for amino acid uptake by mycorrhizal roots collected along a European North/South gradient.

Tree species	Glutamine		Glycine	
	V_{max}	K_m	V_{max}	K_m
	Lactarius subdulcis			
Fagus sylvatica	13.3 ± 1.0	81 ± 14	18.1 ± 1.5	105 ± 18
Fagus sylvatica	11.6 ± 1.2	77 ± 18	14.5 ± 1.1	102 ± 16
Fagus sylvatica	12.4 ± 0.9	79 ± 13	22.8 ± 4.2	197 ± 67
	Russula ochroleuca			
Fagus sylvatica	5.3 ± 0.6	79 ± 21	4.4 ± 0.9	127 ± 49
Picea abies	n.a.	n.a.	4.8 ± 0.6	135 ± 30
Fagus sylvatica	1.4 ± 0.1	86 ± 20	3.1 ± 0.3	233 ± 35
	Unidentified morphotypes			
Pinus sylvestris	11.6 ± 1.6	130 ± 38	8.3 ± 1.0	50 ± 17
Picea abies	7.7 ± 1.4	36 ± 22	10.7 ± 1.8	42 ± 20
Picea abies	24.7 ± 3.1	104 ± 29	n.a.	n.a.
Picea abies	24.0 ± 6.7	111 ± 59	34.1 ± 5.6	113 ± 38
Fagus sylvatica	14.7 ± 1.6	19 ± 8	17.9 ± 1.7	21 ± 7

Values for V_{max} (μmol/g DW/h) and K_m (mmol/m^3) (±SE of the fitted parameters) were calculated by non-linear curve fitting of the experimental data to the Michaelis-Menten equation [$v = (V_{max} \times [S])/(K_m + [S])$] in a substrate concentration range of 0.001–0.25 mol/m^3; n.a. = not available. From Wallenda and Read (1999).

no growth was observed without colonization. However, growth and N concentration were greatly increased in ECM plants. There were some differences in the effectiveness of the three fungi, *H. crustuliniforme* giving higher yields and N contents than *A. muscaria* which was, in turn, more effective than *P. involutus*. Yields and N contents were, in general, higher on the peptides than on the amino acids and, in plants colonized by the two most effective fungi, the high values were obtained with the largest peptide units (Figure 9.7).

Plants colonized by those ECM fungi which can hydrolyse BSA and gliadin show increases in growth and N content when grown with these proteins as sole N source. Thus, yields of *Pinus contorta* colonized by *R. roseolus* or *S. bovinus* were significantly higher than those of non-mycorrhizal plants supplied with BSA and were similar to those grown with ammonium at the same N concentration. Colonization by *P. tinctorius*, shown in pure culture to have lower proteolytic ability, gave little access to the substrate and the plants grew less well (Abuzinadah *et al.*, 1986; Figure 9.8a, b). The 'protein fungus' *H. crustuliniforme* gave similar responses in *B. pendula*, *P. sitchensis* and *P. contorta*, while none of these species had access to protein N without colonization

Figure 9.7 Growth (G) and nitrogen contents (N) of roots (open bar), shoots (diagonally hatched bars) and whole plants (vertically hatched bars) of *Betula pendula* colonized by *Hebeloma crustuliniforme* (HC), *Amanita muscaria* (AM), *Paxillus involutus* (PI), or non-colonized (NM) grown with different amino acids and peptides as sole N sources for 75 days. Vertical bars represent least significant difference (LSD) for roots (R), shoots (Sh) and whole plants (PI). (a) Alanine; (b) di-alanine; (c) penta-alanine; (d) hexa-alanine. From Abuzinadah and Read (1986b), with permission.

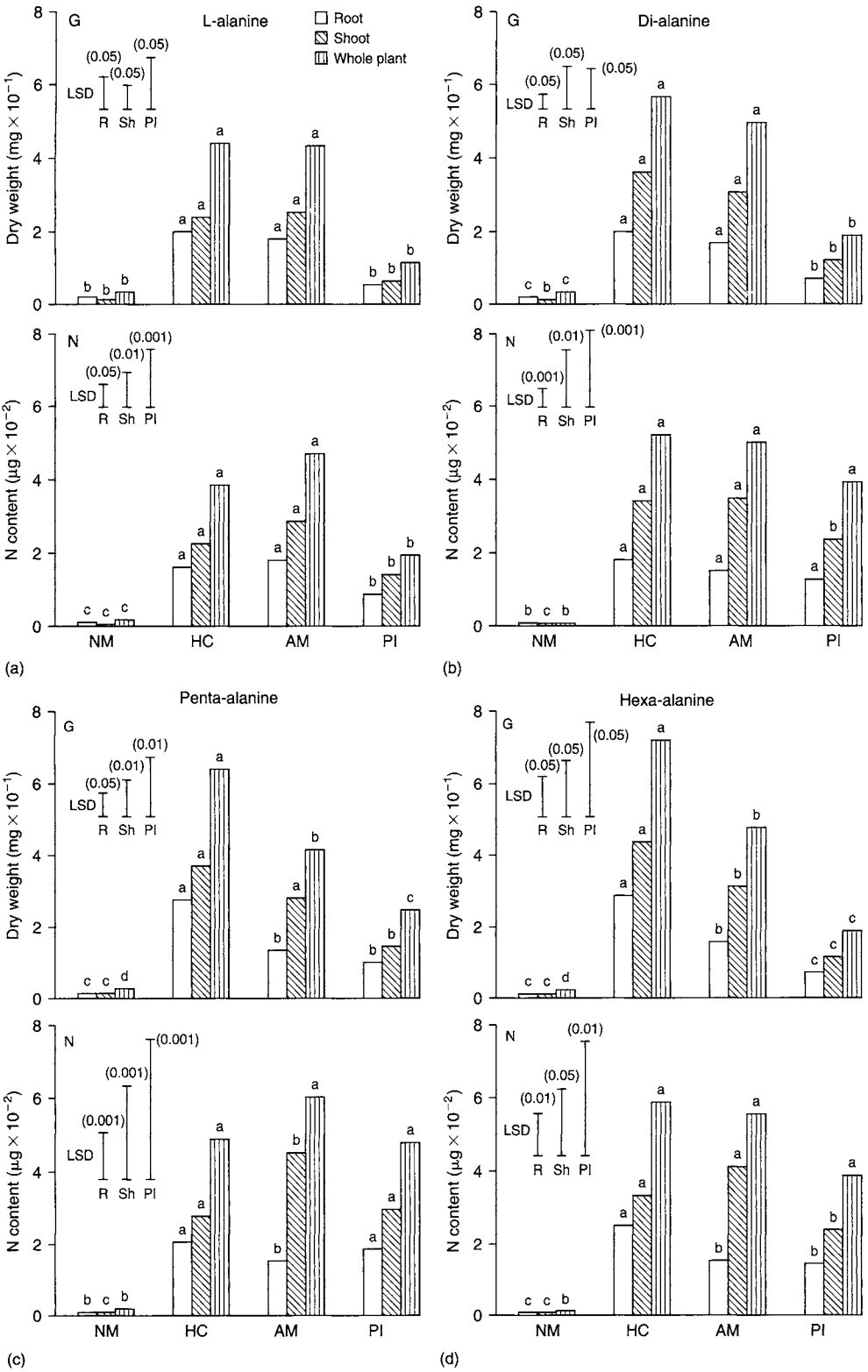

Figure 9.7 (Caption on p. 341)

(Abuzinadah and Read, 1986b). ECM colonization also had a major impact upon N utilization by *Eucalyptus* seedlings (Turnbull *et al.*, 1995). Both *E. grandis* and *E. maculata* used amino acids and protein when they were ECM, an ability lacking in non-mycorrhizal seedlings. Both species grew more than non-mycorrhizal plants on protein as well as on the amino acids arginine, asparagine and histidine, when colonized by an *Elaphomyces* spp. (Figure 9.9a). Although both species used protein less readily when colonized by a *Pisolithus* spp. (Figure 9.9b), the ECM plants were still significantly larger that their non-colonized counterparts. When *Betula* was grown monoxenically with selected mycorrhizal or saprotrophic fungi, colonization by *A. muscaria* and *H. crustuliniforme* gave highest yields and N content on protein N, while *P. involutus* was somewhat less effective as a symbiont (Abuzinadah and Read, 1988). In the presence of a seed-borne saprotroph, *Ulocladium botrytis,* or an ERM fungus, *Rhizoscyphus ericae,* no growth responses or net increases of N content of the *Betula* were observed. In contrast, inoculation with the saprotroph *Oidiodendron griseum,* while giving lower plant yields and total N contents than *A. muscaria* or *H. crustuliniforme,* did facilitate accumulation of N in the tissues of *Betula,* presumably because N became available via mineralization (Abuzinadah and Read, 1989b).

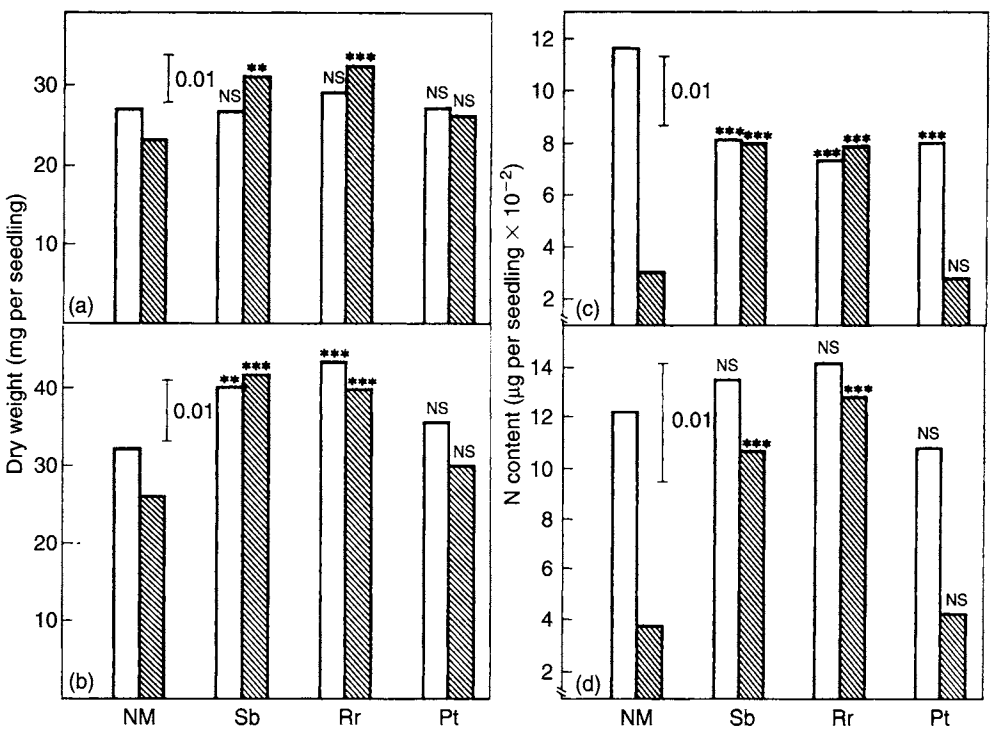

Figure 9.8 Dry weights (a and b) and nitrogen contents (c and d) of *Pinus contorta* colonized by *Suillus bovinus* (Sb), *Rhizopogon roseolus* (Rr) or *Pisolithus tinctorius* (Pt), or non-mycorrhizal (NM) after 40 (a and c) or 80 (b and d) days growth on ammonium (open bars) or bovine serum albumin (hatched bars) as sole N source. Vertical bars represent least significant difference (LSD) and asterisks indicate significant differences between mycorrhizal and non-mycorrhizal plants within each treatment. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS, not significant. From Abuzinadah *et al.* (1986), with permission.

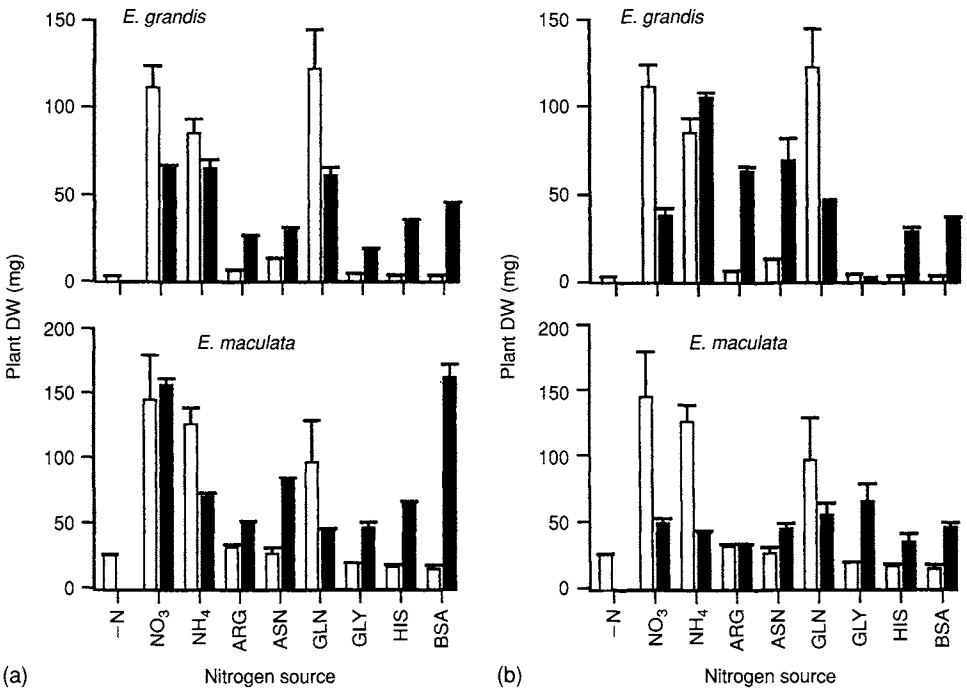

Figure 9.9 Growth of seedlings of *Eucalyptus grandis* or *E. maculata* in agriculture on a range of inorganic and organic N sources, either non-mycorrhizal (open bars) or ectomycorrhizal (closed bars) with (a) *Elaphomyces* spp. or (b) *Pisolithus* spp. Data are means of 12 replicate plants, ± standard errors of means. From Turnbull *et al.* (1995), with permission.

These results highlight one of the difficulties in extrapolating from experiments carried out under monoxenic conditions. In this case, a non-mycorrhizal saprotroph presumably converted protein to a form which could be absorbed and assimilated by the plant. In a mixed population of heterotrophs, such products are less likely to accumulate. Clearly, the extent of benefit accruing to the plant from its fungal associates will depend upon a combination of factors which include plant–fungus compatibility, the ability of the symbiont to compete with other soil or rhizosphere organisms for a resource and, having gained access to a substrate, its capacity to mobilize the nutrients which it contains. These factors can only be realistically evaluated in the presence of mixed natural populations of symbionts and saprotrophs, although under these conditions the mechanisms operating will be hard to unravel.

Dighton *et al.* (1987) approached this problem by examining the breakdown of the N-containing substrates powdered animal hide and chitin by ECM plants of *P. contorta*, grown in the presence or absence of the basidiomycete decomposer *Mycena galopus*. When roots of the plant were colonized by the ECM fungi *H. crustuliniforme* or *Suillus luteus*, significantly greater degradation of these substrates occurred, together with enhanced plant growth. In the presence of the saprotroph, the effectiveness of the ECM fungi in acquiring N was much reduced. However, interpretation of these results is complicated by the addition of readily available C to the medium, thus increasing the C:N ratio and probably enhancing, to an unrealistic extent, the ability of the

saprotroph to compete for N in the organic substrates. Indeed, one of the advantages which all mycorrhizal fungi may have is that (in symbiosis) they are not dependent for C on organic substrates in soil. In consequence, the C:N (or C:P etc.) ratio of the substrates will be considerably less important for them than for saprotrophs.

Ultimately evaluation of the role of ECM fungi in mobilization of N under natural conditions can be achieved only by supplying colonized plants with the substrates which they would normally encounter in the field, in the presence of a natural population of soil microorganisms. Bending and Read (1995a) grew plants of *Pinus sylvestris* colonized by *S. bovinus* or *Thelephora terrestris* on non-sterile peat in transparent observation chambers. Organic matter freshly collected from the fermentation horizon (FH) of a pine forest was supplied in weighed aliquots on plastic trays. On reaching such introduced organic materials, the mycelia of the ECM fungi typically proliferate intensively to form dense 'patches' over the substrates (see Colour Plate 6.2c, d). After a standardized period of 'occupation' of the organic matter, the total N content of the material was measured and the quantities of N mineralized during the incubation period determined. There was no significant loss of N in the controls, whereas in the organic matter colonized by both fungi there was a significant (23%) depletion of N in material colonized by *S. bovinus* and 13% in that colonized by *T. terrestris* (Figure 9.10). These values are similar to those obtained by Entry *et al.* (1991b) who measured decline of N concentration in litter of *Pseudotsuga menziesii* colonized by mycelia of the mat-forming mycorrhizal fungus *Hysterangium setchelli* in the field. Over one year they observed losses of 32% of initial N from the litter. In an experiment involving supply of litter obtained from different tree species and using *Paxillus involutus* as the mycobiont of *Betula pendula*, it was confirmed that ECM colonization of the litter resulted in improved plant yield, but patterns of exploitation of the individual nutrients were different from those reported by Bending and Read (1995a) using different ECM fungal partners (Perez-Moreno and Read, 2000). In only one litter type was the loss of N more than 10% and, whereas whole seedling N content was increased by colonization, tissue N concentrations were not significantly different in ECM and non-mycorrhizal plants. In this experiment, litter P was exploited by the ECM plants more effectively than N. The different patterns of nutrient exploitation in the two experiments probably arose at least in part from the use of different fungal symbionts. Thus, apart from the broad conclusion that ECM colonization facilitates mobilization and removal of nutrients from organic residues of the FH, further generalizations from observations involving particular plant–fungal partnerships should be avoided.

Experiments examining plant litter as a potential source of N for ECM plants have been followed by studies in which individual components of typical boreal forest mycorrhizospheres are provided as substrates. Among these, pollen (Perez-Moreno and Read, 2001a), seeds (Tibbett and Sanders, 2002), nematodes (Perez-Moreno and Rea, 2001b) and collembolans (Klironomos and Hart, 2001) have been selected on the basis that, during parts of the growing season at least, the cellular contents of these materials represent large and rapidly turning over potential sources of nutrients (see Chapter 15). In the case of pollen, the N contained in weighed aliquots of the substrate was reduced by 76% when supplied to plants grown in symbiosis with *P. involutus*, whereas in non-mycorrhizal systems only 42% was removed, presumably by saprotrophs, over the same period. Whereas a large proportion of the exported N was found in the ECM plants, the non-mycorrhizal plants gained relatively small amounts

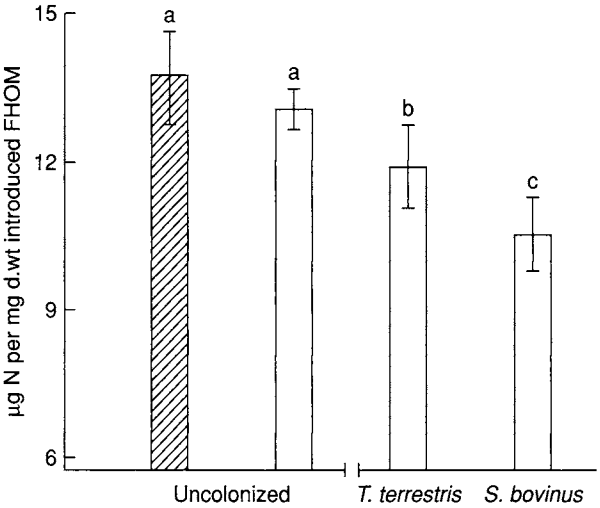

Figure 9.10 Utilization of N from litter collected from the FH horizon of forest soil. The hatched bar shows N content at the start of the experiment and open bars show N content after incubation, uncolonized or colonized by mycelium of *Thelephora terrestris* or *Suillus bovinus*, growing in symbiosis with *Pinus sylvestris*. FHOM = fermentation horizon organic matter. Treatments with different letters are significantly different at $P < 0.01$. From Bending and Read (1995a), with permission.

of the nutrient. When air-dried nematode cadavers were supplied as sole additional sources of N in the same way, 68% of the N was removed in the ECM systems and only 37% in those lacking a fungal symbiont (Perez-Moreno and Read, 2001b).

As yet we know little about the metabolic processes involved in the mobilization of N in naturally occurring organic residues. Their colonization by mycorrhizal fungi both in the field (Griffiths and Caldwell, 1992) and in microcosms (Bending and Read, 1995b) leads to significant increases of proteolytic activity. These observations, coupled with those showing proteolytic capability of the fungi in axenic culture and on colonized plants under aseptic conditions, are strongly suggestive of a direct role of ECM associations in mobilizing N from natural substrates, but do not provide definitive evidence of this. An alternative explanation is that the presence of ECM mycelium in the substrates somehow facilitates the activities of saprotrophs. What is evident is that the ECM fungi, by intensive colonization of the substrate and provision of organized mycelial aggregates connecting resource deposits to roots, are, in many cases, extremely effective as scavengers for, and transporters of, nitrogenous materials.

Conclusions

The ease with which many ECM fungi can be grown in axenic culture has enabled extensive screening of their abilities to use different forms of N. Most species readily use ammonium, nitrate and some simple organic-N compounds, although there are differences at both the inter- and intraspecific levels. Much new information has been gained concerning the biochemistry of N assimilation, in particular in relation to the enzymes involved in assimilation of ammonium which, in many ECM

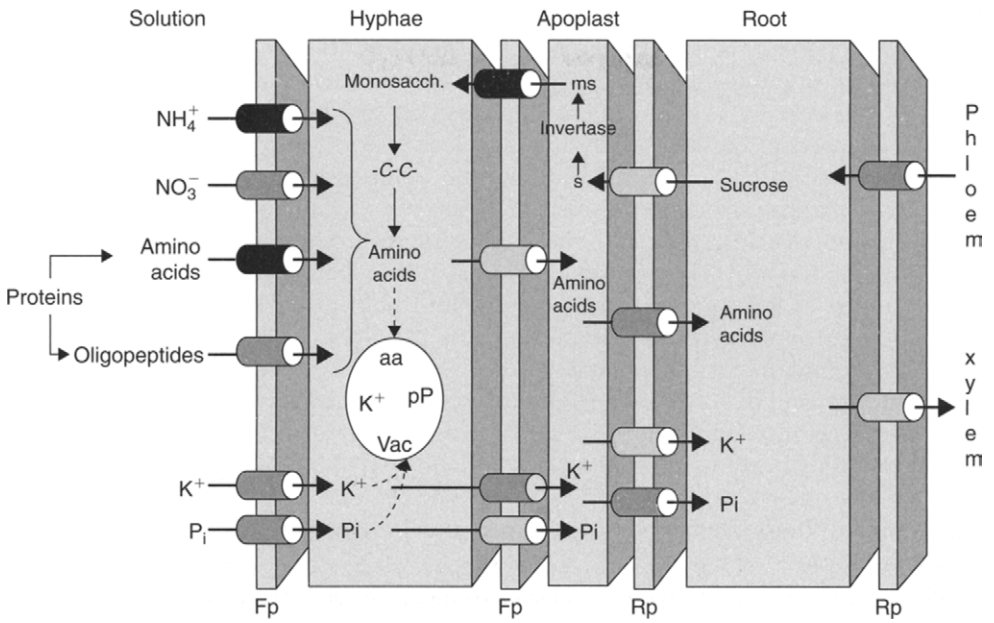

Figure 9.11 Diagrammatic representation of the current understanding of the location and function of nitrogen transporters in ectomycorrhizal tissues. Black cylinders represent structures in which at least one member of the transporter family has been fully characterized by functional complementation in a deficicent strain of yeast. Dark grey cylinders represent putative transporters in which candidate genes have been identified as expressed sequence tags. Pale grey cylinders represent hypothetical transporters. The transporters putatively involved in carbohydrate transport, carboxylation of N compounds and in transfer of phosphorus (P) and potassium (K^+) ions are also shown. Fp, fungal plasma membrane; Rp, root plasma membrane; aa, amino acids; pP, polyphosphate; Vac, vacuole; s, sucrose; ms, monosaccharide; cc, carboxylation. Modified from Chalot *et al.* (2002), with permission.

fungi, appears to be the preferred source of inorganic N. These studies have been extended to allow analysis of N assimilation by the fungi when grown in symbiosis with plants and, importantly, to investigate the influence of the plants themselves upon the pattern of events. Progress has been made towards characterization of the processes of nitrate and ammonium uptake and transport at the molecular levels. This has enabled a better understanding of the mechanisms and pathways whereby uptake of mineral N sources takes place in ECM symbioses.

Increasingly, it has been recognized that much of the N contained in the superficial layer of soil occupied by ECM roots is in organic form and that some ECM fungi have access to these more complex N sources. Emphasis has turned on the one hand to characterization of the primary sources of organic N in forest soils, with consideration of the extent to which these are accessible to ECM fungi and, on the other, to consideration of the molecular and biochemical events involved in the uptake, assimilation and transport of organic N compounds.

The molecular studies, mostly using *Amanita muscaria* (Nehls *et al.*, 1999) or *Hebeloma* spp. (Javelle *et al.*, 2001; Wipf *et al.*, 2002; Selle *et al.*, 2005; Wright *et al.*, 2005;

Benjdia *et al.*, 2006), by revealing N dependent expression profiles of N importer genes, have enabled us to envisage how N uptake by the fungi and transfer to the plant may occur in nature (Figure 9.11). Expression of these genes is likely to be regulated by the internal N status of the hyphae. This will be relatively reduced when they are exposed to the essentially low ambient N concentrations of the soil solution, but higher in the N-accumulating mantle tissues surrounding the root. Under these circumstances strong expression of N importers in the soil compartment can be expected. Conversely, their activities will be repressed in the hyphae of the Hartig net adjacent to the root. This repression would inhibit reabsorption of N compounds by the fungus at the fungus–plant interface and help to facilitate the export of N either in the form of amino acid or ammonia, to the plant.

It is important to recognize that the pathways and processes involved in the import and transfer of N across the ECM interfaces are intrinsically linked to those by which carbon is transferred in the reverse direction (Figure 9.11 and see Chapter 8). In addition, the ability to capture and assimilate N will be influenced by the availability of the other macronutrients, phosphorus (P) and potassium (K). The processes whereby these elements are acquired and transported are considered in the following chapter.