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Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas

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Abstract

Ectomycorrhizal fungi are symbiotically associated microorganisms which ecological importance has been repeatedly demonstrated. There has been a considerable amount of research aimed at assessing the ability of ectomycorrhizal fungi and ectomycorrhizas to utilize organic nitrogen sources. The fate of soil proteins, peptides and amino acids has been studied from a number of perspectives. Exocellular hydrolytic enzymes have been detected and characterized in a number of ectomycorrhizal and ericoid fungi. Studies on amino acid transport through the plasma membrane have demonstrated the ability of ectomycorrhizal fungi to take up the products of proteolytic activities. Investigations on intracellular metabolism of amino acids have allowed the identification of the metabolic pathways involved. Possible intracellular compartmentation of amino acids will be examined by immunocytochemistry. Further translocation of amino acids in symbiotic tissues has been established by experiments using isotopic tracers, although the exact nature of the nitrogenous compounds transferred at the symbiotic interface remained unclear. One of the main future challenges in the physiology of organic nitrogen acquisition is to determine the nature, the regulation and the location of N-compound transporters at the soil-fungus and fungus-plant interfaces. The molecular approach which is just emerging in this particular research area will greatly improve our knowledge. Future research should also address the extent of competition between different ectomycorrhizal species and between different microbial populations for organic nitrogen.

Keywords: Carbon metabolism; Nitrogen metabolism; Ectomycorrhizal fungus; Ectomycorrhiza; Hydrolytic enzyme activity; Organic nitrogen; Transport system

Contents

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1. Introduction

Nitrogen plays a critical role in plant biochemistry, being an integral component of many compounds, including chlorophyll and enzymes, amino acids and proteins, all of which are essential for plant growth processes. Nitrogen is essential for carbohydrate use within plants and stimulates root growth and development as well as the uptake of other nutrients.

However, N is the most commonly limiting nutrient in terrestrial ecosystems. In forest ecosystems, the exchangeable N in the soil solution – that is, soil ammonium and nitrate loosely held on the soil colloids by electrostatic forces and immediately available to plants and microbes – is very low relative to total soil N [1] and most of the soil nitrogen is in organic form. However, proteins and other organic nitrogen compounds are bound in recalcitrant forms of organic matter or are chemically fixed within clays, which protect them from rapid microbial breakdown. Thus even though soil N normally exceeds plant N by many times, N deficiencies in natural ecosystems are common because only a small fraction of total soil N is available to plants at any given time. Competition among decomposers, nitrifying bacteria, non-biological processes, and plants for this nutrient is intense and plants have therefore adapted strategies for efficient uptake and recycling of nitrogen [2]. One of these strategies is the formation of ectomycorrhizas which allows woody plants to compete efficiently with soil microorganisms [3,4]. Ectomycorrhizal fungi are symbiotically associated with the roots of many woody plants and can dramatically enhance plant growth. George and Marschner [5] suggested that, in forest soils with high organic matter content, the much better acquisition of organically bound nitrogen by ectomycorrhizal roots may be the reason for improved nitrogen nutrition, rather than the increased uptake of mineral nitrogen. Experiments by Read and his co-workers during the past 12 years unequivocally support Frank's organic nitrogen theory, that mycorrhizal infection might provide access to the nitrogenous reserves contained in organic horizons [6].

Turnbull [7] demonstrated that mycorrhizal asso-

ciations confer on *Eucalyptus* species the ability to broaden their nitrogen resources. Kaye and Hart [8] recently presented a summary of studies showing substantial plant uptake of organic N. Most of the plants listed in their review were mycorrhizal species. On the basis of comparative uptake measurements, it was shown that amino acid absorption by plants of heathland and arctic tundra may account for 10–82% of the total plant N uptake, depending on the species [9].

Melin and Nilsson [10,11] first demonstrated the role of the mycorrhizal mycelium in the translocation of ^{15}N-labelled nitrogen compounds to colonized plants. Indeed, both VA and ectomycorrhizal plants have specialized hyphae which are termed respectively 'runner hyphae' [12] and rhizomorphs to transport nutrients and water and to promote mycelial networks. Further transfer of nutrients from the fungus to the host takes place through the symbiotic interface and comprehensive reviews have been given recently [13–15]. Transfer cell structures within ectomycorrhizas have been described [16,17]. The organization of the Hartig net in mature mycorrhizal roots formed by *Amanita muscaria* and *Picea abies* has been studied [16]. The authors observed the lack of septate hyphae and their intimate juxtaposition, which results in a coenocytic, transfer cell-like structure of the Hartig net. The lack of septa should facilitate interhyphal transport. Wall ingrowths of *Pisonia* mycorrhiza resemble those that develop in transfer cells of other plant species at sites where high rates of nutrient transfer between the apoplast and symplast may be predicted [17].

Hadas et al. [18] hypothesized that transfer of N into microbial biomass during the decay of organic matter may follow two patterns: (i) microorganisms directly incorporate low molecular mass organic N compounds, released from the breakdown of organic matter, and assimilate the amino groups; (ii) microorganisms assimilate inorganic N resulting from mineralization of organic N. To test the relative importance of these two processes, equal concentrations of NH_4^+-N and alanine-N were added to soils. Results showed that both pathways operated concurrently [18]. However, care must be taken before generalizations are made since these experiments were performed with soils of pH greater than 7.5.

Inorganic nitrogen assimilation has been extensively studied in a number of fungal and symbiotic models described in recent reviews [4,19,20] whereas less has been done concerning the direct incorporation of organic N compounds into fungal cells. Data from Harley [21] and Carrodus [22] suggested that glutamine was an early product of ammonium assimilation by beech mycorrhizas. ^{15}N feeding procedures have subsequently demonstrated unequivocally that glutamine is the main sink for nitrogen in the assimilation of ammonium by beech, pine and spruce ectomycorrhizas [23–26]. Furthermore, there is evidence that aspartate, asparagine and alanine are important intermediates in the assimilation of nitrogen in the fungus-root complexes [23,24,26,27] suggesting high activities of aminotransferases.

The main purpose of this review is to summarize present knowledge on organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas and to highlight recent physiological and biochemical data which have led us to consider organic nitrogen as an essential source of nitrogen for ectomycorrhizal fungi and ectomycorrhizas. The first section reviews the exocellular degradation of macromolecular nitrogen, including data on exocellular hydrolytic enzymes. Studies on ericoid endophytes are mostly included in this section for comparisons with ectomycorrhizal fungi. In the second section, the discussion will concentrate on the transport mechanisms involved in the acquisition of organic nitrogen by mycorrhizal fungi, including its subsequent transfer in symbiotic ectomycorrhizal associations. In the third section, different aspects of intracellular degradation of organic nitrogen, with particular attention to amino acids, will be outlined. Detailed enzymatic pathways will not be presented in the present review. The potential use of immunocytochemistry for direct localization of amino acids in fungal cells is reviewed in the last section.

2. Extracellular degradation of organic nitrogen compounds

Much of the nitrogen added to the soil undergoes many transformations before it is removed. The cycling of nitrogen in forest soils has been extensively reviewed [1,28,29] and will not be dealt with in detail

here. This section will rather concentrate on the available forms of organic nitrogen in forest soils and their extracellular degradation by mycorrhizal fungi.

2.1. Forms of organic nitrogen available in forest soils

Organic nitrogen is the predominant form of N in most temperate forest soils [30,31]. In one calcareous and two acid forest soils the proportion of organic N was more than 95% of the total [30]. In the same study, results demonstrated that a significant part of the applied ^{15}N ammonium was incorporated into organic nitrogen compounds. Other studies have shown that organic nitrogen comprised a large proportion of the total N in soil solution in a young mixed-conifer forest [32]. Mineral nitrogen contents were estimated in various forests, both in the soil and in the litter. For instance, NH_4^+ and NO_3^- amounted to less than 5% of total N in the soil solution isolated from ectomycorrhizal mat soils [33]. Organic forms of nitrogen contributed 51 and 61% respectively to total soluble water extractable soil nitrogen in *Eucalyptus grandis* and *Eucalyptus maculata* forest soils [7]. However, caution must be used when interpreting data on nutrient soil contents since under many circumstances in nature the N cycle is not a steady state, and pool sizes are constantly changing [28].

There have been very few quantitative studies of the status of forest soils with respect to organic nitrogen compounds. Among such compounds, most attention has been paid to free amino acids. A number of studies have provided evidence that a broad spectrum of individual amino acids can be present in 'free' form in acid organic soils in forest ecosystems [34,35]. Qualitative and quantitative aspects of the distribution of individual amino acids have been assessed [35]. Amino acid nitrogen comprised the most important component of this hydrolyzable fraction, being equivalent to 38.8% of the total N in the uppermost horizon and 22.8% in the lowest. However, amino acids accounted for only 3.3% and 1.8% of the total soluble water extractable soil nitrogen in an *Eucalyptus grandis* and *Eucalyptus maculata* forest soil, respectively [7]. The movement of a given amino acid in a soil solution will be determined by its concentration together with its diffusion coefficient and the buffering capacity of the soil. This is important since, in the field, the diffusion of ions to the root surface also controls nutrient supply [36]. The neutral and acidic amino acids (which exist primarily as anions since the protonated amino group does not fully dissociate below pH 9) probably have similar mobility to that of ammonium whereas the basic amino acids (arginine and lysine) would tend to be bound more tightly to the cation exchange complex [9]. Not surprisingly, alanine, glutamic acid, aspartic acid, leucine, valine, serine, glycine are found to be important amino acids in most studies, with concentrations ranging from 1 to 10 μg per g dry soil.

However, organic nitrogen compounds other than free amino acids such as combined amino acids (i.e. peptides and proteins) and nucleic acids may also be quantitatively important to ectomycorrhizal fungi. Incubation of material from the floor of mountain beech forest with inorganic ^{15}N resulted in nuclear magnetic resonance spectra dominated by peaks assigned to peptides (80% of the total signal intensity) and nucleic acids (12%) [37].

2.2. Enzymes involved in mobilization of macromolecular nitrogen

Ectomycorrhizal mycelium constitutes a large proportion of the microbial biomass in many forest soils [38,39] and an even higher proportion in ectomycorrhizal mat communities [31,40]. The ability of ectomycorrhizal fungi to degrade macromolecular nitrogen and furthermore to take up and assimilate the products of hydrolytic degradation is therefore likely to have a great influence on the dynamics of organic N utilization in forest ecosystems. A number of observations support the view that ectomycorrhizal fungi are directly involved in the mobilization of nitrogen from organic matter. All of the major hydrolytic enzymes involved in mobilization of nitrogen from organic compounds (Table 1) have been detected in ericoid and some in ectomycorrhizal fungi [41].

The ability of ericoid and ectomycorrhizal fungi to use protein as a major growth substrate is correlated with production of exocellular proteinase activities [42–46]. The ability of several ectomycorrhizal fungi to assimilate protein and to transfer its nitrogen to plants of *Pinus contorta* was demonstrated [47,48].

Table 1
Induction and characteristics of enzymes involved in mobilization of macromolecular nitrogen by ectomycorrhizal and ericoid fungi and by ectomycorrhizas (adapted and extended from [41])

Organism/Reference	Conditions for induction	Characteristics
Proteolytic enzymes		
S. variegatus	Growth on ammonium	Detected in mycelial extracts and culture filtrates
S. bovinus		Strong activity against peptides
P. croceum		Serine type protease
P. tinctorius [58]		
H. ericae [42,43]	Growth on liquid or solid media containing BSA or in symbiosis	Acid proteinase
	Optimum pH for induction = 4	Optimum pH for activity = 2.2 Inhibition by pepstatin K_m = 1.4 μM for FITC-BSA Glucose repression with BSA
C. geophilum	Growth on BSA, gelatine, casein or on litter proteins	Acid protease purified from *C. geophilum* and *A. rubescens* induced by litter proteins
H. crustuliniforme	Optimum pH for induction = 4	Optimum pH = 5
A. rubescens		MM = 72 kDa
L. subdulcis [20,59]		
H. crustuliniforme [60,61]	Growth on liquid media containing BSA Optimum pH for induction = 3	Acid proteinase (MM = 38 kDa) Inhibition by pepstatin Optimum pH for activity = 2.5 No activity against peptides Optimum induction with BSA and glucose
P. involutus [66]	Colonization of organic matter (birch horizon) by ECM fungi	Optimum pH = 3.5–4.5 and 7
T. terrestris	Colonization of beech leaf litter bags by ECM fungi	Low induction by ECM fungi compared to saprophytic fungi
S. bovinus		Glucose repression
L. nuda [63]		Lack of inducers
Chitinase		
H. ericae [44,50]	Pure chitin Optimum pH for induction = 4.5–6.0	Exocellular chitinase activity
Ribonuclease		
Mat-forming ectomycorrhizal fungi [54]	RNA	Hydrolysis of phosphodiester substrates

Conversely, the ability of four major species of trees to utilize protein as a sole nitrogen source when associated to a single mycorrhizal fungus was proved [49].

Chitin is another potential polymeric source of N and its degradation by the ericoid mycorrhizal fungus *Hymenoscyphus ericae* has been investigated [50,51]. Chitinolytic activity was found to be induced in *H. ericae* when chitin was the sole N source [50]. It was recently further demonstrated that significant quantities of N derived from chitin can be transferred to the host plant [51]. It is also relevant to note that chitin may serve as a carbon source for fungi, e.g. for the ectomycorrhizal fungus *Paxillus involutus*, although it is still unclear how it is degraded [52].

Fokin et al. [53] concluded that microorganisms from a podzolic soil play a major role in the decomposition of nucleic bases (uracil). Ribonuclease activities have been detected in mat-forming ectomycorrhizal fungi [54] and *H. ericae* was shown to rapidly hydrolyze DNA, thus giving its host the capacity for using nuclei as a source of phosphorus [55]. In the latter study, there were indirect proofs of N utilization from nuclei but direct utilization of the nitrogen from nucleic acids by ectomycorrhizal fungi and their ecological relevance for nitrogen nutrition have not been investigated so far. Such studies

have been extensively carried out on marine microbial populations (for references, see [56]).

2.2.1. Occurrence of proteolytic enzymes

Exocellular protease activity is considered to be important in the production of oligopeptides from proteins, resulting in the subsequent release of low molecular-mass compounds which are assimilated by indigenous flora [57]. The induction, production and characterization of exocellular proteinase activities have been studied both in ericoid and ectomycorrhizal fungi [44–46,58–61]. These experiments confirmed earlier results which demonstrated proteinase activity in a number of mycorrhiza-forming species [62]. However, the author concluded that mycorrhiza-forming species were clearly inferior to the litter-decomposers in the production of enzymes. Similarly, protease activity was found to be much higher in the beech litter inoculated with the saprotrophic basidiomycete *Lepista nuda* (1.54 units g^{-1} d. wt., 13 weeks after inoculation), when compared with the beech litter inoculated with the mycorrhizal fungus *Thelephora terrestris* (0.08 units g^{-1} d. wt.) [63]. This seems surprising since the frequency of occurrence of mycorrhizal fungi was found to be greater in soils of high organic matter content whereas saprophytic fungi were more common in soils of lower organic matter content [64]. However, protease activities were assayed either in pure culture fungi [62] or litter bags colonized by fungal species [63] and therefore in situ measurements of protease activity for both ectomycorrhizal and saprophytic fungi are needed to clarify this point.

Recent experiments have proved that the abilities of ectomycorrhizal fungi to mobilize nitrogen from soils are also expressed in the natural environment [65]. These degradative capacities are associated with an increase in protease activity during colonization of organic matter [66]. It has also been demonstrated that the inducible protease activity in the ectomycorrhizal fungus *Amanita rubescens* was 6-fold higher in a colony grown with a protein extracted from a beech forest litter, as compared with bovine serum albumin (BSA) or gelatine [20]. *Amanita rubescens* and *Lactarius subdulcis*, which are usually associated with organic horizons, secreted larger amounts of proteases than *Cenococcum geophilum* and *Hebeloma cylindrosporum*, which colonize predominantly mineral soil layers, reflecting to some extent the distribution of the ectomycorrhizal fungi in their natural environment. Several mat-forming ectomycorrhizal fungi were found to be capable of growing on an insoluble tannic acid-protein complex suggesting that they are able to overcome the problem of protein immobilization [54]. The abilities of mycorrhizal fungi to use protein-tannin complexes may turn out to be a key reaction in acid, nutrient poor soils, as in heathlands and arctic systems. However, a low induction of protease activity in a litter colonized by the ectomycorrhizal fungus *Suillus bovinus* was established despite the N-limiting conditions [63]. The authors attributed this lack of induction to the low level of inducers and/or to glucose repression which might have restricted the supply of energy.

2.2.2. Properties of proteolytic enzymes

In ericoid endophytes and ectomycorrhizal fungi, production of exocellular proteinases clearly depends on the pH of the environment. Maximum proteinase production by ericoid mycorrhizal fungi has been shown to occur at a culture pH of 4.0–5.0 [44]. Botton and Chalot [20] noticed that the optimum pH for protease activity induced by supplying pure cultures of *Cenococcum geophilum* and *Amanita rubescens* or beech ectomycorrhizas with proteins extracted from leaf litter was about 5. In addition an alkaline proteasic activity, detected when FITC-BSA was used as the assay substrate, was induced in *Cenococcum geophilum* when colonies were supplied with BSA, casein or gelatine as the sole N source [59]. However, the role of alkaline protease remains unclear since most ectomycorrhizal fungi cannot use proteins in environments of neutral pH [44,47]. The soil pH therefore seems to be a key factor controlling the incorporation of organic matter into the microbial biomass, as previously stated by Wardle in his review paper [67]. It may affect both proteinase production [20,44,47] and amino acid uptake capacities [68] by mycorrhizal fungi.

The specificity of proteases towards their substrates has been investigated in the ectomycorrhizal fungus *Cenococcum geophilum* [59]. The proteolytic activity was strongly inhibited by two protease inhibitors, diisopropyl fluorophosphate (DIFP) and phenylmethylsulfonyl fluoride (PMSF), which suggests

that the proteases isolated from this fungus were serine proteases. This was further demonstrated by the use of *p*-nitroanilide-labelled peptides as substrates. The highest activity was found with a peptide exhibiting a tyrosine residue, which is the target amino acid for serine proteases.

3. Uptake of organic nitrogen compounds

Both plants and fungi can absorb a range of relatively simple organic N compounds [69]. However, plants rarely get the opportunity to do so because their roots are at a spatial disadvantage in competition with microorganisms, a wide range of which use such compounds both as nitrogen and energy sources. In symbiotic associations such as mycorrhizas the location of the symbiosis ensures that mycelial growth of the fungus into soil provides access to nutrients in the soil solution.

3.1. General features of membrane transport in fungi

Almost all knowledge about membrane transport in fungi derived from studies with only two species: *Saccharomyces cerevisiae* and *Neurospora crassa*. This is 0.0001% of the total estimated number of species. Furthermore, only 0.03% of the total species of the Basidiomycetes has been investigated for membrane transport [70]. Jennings [71] suggested that uptake systems of filamentous fungi are highly efficient for scavenging the environment to maximize whatever nutrients are available. More recently Burgstaller [70] postulated that the properties of plasma membrane transport are closely associated with the habitat and with the ecological role of filamentous fungi. Permeability of biological membranes is quite restricted, resulting in most of the cellular nutrients entering the cell via specific systems: channels or transporters (also named permeases or carriers). The only formal distinction between channels and carriers is that the solute binding sites of a channel are accessible from both sides of the membrane at the same time whereas the binding site of a carrier protein is, at a given time, either accessible from one side of the membrane or from the other, but not from both [72]. The following section will deal with the description of transport systems for nitrogenous compounds in mycorrhizal fungi for which information is just emerging.

3.2. Transport of organic nitrogen by pure culture fungi

3.2.1. Transport of amino acids

Marschner and Dell [73] have estimated that in spruce ectomycorrhizas, more than 90% of the root apical zones are enclosed by a fungal sheath. Moreover, it was unequivocally calculated that the external mycelium of *Pisolithus tinctorius* and *Cenococcum geophilum* inoculated pine comprised 75% of the potential absorbing surface area while it accounted for only 5% of the dry weight [74]. These facts highlight the need for more studies on uptake capacities by fungal tissues. Amino acids do not permeate freely through the plasma membrane and require transporters for their uptake. Comprehensive reviews of amino acid transport systems in fungi, yeasts, and higher plants have been recently given [75–83] and will not be dealt with in detail in the present review. In the following section, the main features of amino acid transporters in fungal cells will be mentioned and thereafter the characteristics of a general amino acid transporter in the ectomycorrhizal fungus *Paxillus involutus* described.

Amino acid transport systems of fungi can generally be grouped into those specific for one or several structurally related amino acids or a general system shared by many amino acids regardless of structure. One of the most difficult tasks in the area of amino acid transport is to differentiate these multiple transport systems and study their characteristics individually because the substrate specificities of these transport systems overlap with each other to a considerable extent in many cases. In addition, amino acids may be transported across the plasma membrane as well as the tonoplast, but these two membranes possess different sets of transport systems, thus adding to the complexity of the overall process of amino acid assimilation. The number of transporters varies between species, from five in *Neurospora crassa* to 16 in *Saccharomyces cerevisiae* [71,75,76,84]. It is however widely accepted that amino acids are transported by H^+ symport mechanisms.

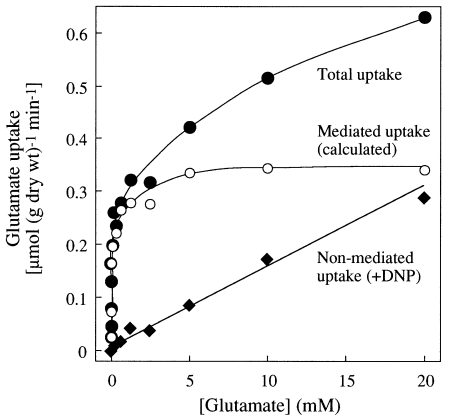

Fig. 1. Effect of substrate concentration on the uptake of glutamate in the absence and presence of dinitrophenol (DNP) by *P. involutus*. Uptake of [^{14}C]glutamate from 0.0024–20 mM solutions was assayed alone or in the presence of 10 μM DNP in the standard conditions. Uptake occurring in the presence of DNP (♦) was subtracted from uptake in non-inhibited conditions (●) for each glutamate concentration, to give the mediated uptake (○).

Carriers have a lot in common with enzymes, and direct correlation in energetic and kinetic terms shows that the basic principle of their catalytic cycle is closely related: (a) binding of substrate, (b) chemical transformation (enzyme) or translocation (carrier), and (c) product release. The main difference is the scalar (chemical) reaction for enzyme, which is vectorial for carrier proteins. This comparative approach has led to significant progress in understanding the energetics of carrier-catalyzed transport. The kinetics, energetics and specificity of a general amino acid transporter have been recently studied in the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. [68,85]. The concentration dependence data of the uptake rate did not obey the simple Michaelis-Menten relationship (Fig. 1), but rather included two components, a saturable carrier-mediated uptake and a non-saturable, diffusion-like process. Kinetic parameters were calculated and were compared to those reported for the uptake of glutamate by other organisms (Table 2). The determination of kinetic parameters may be of importance for studies of competition between organisms in a given environment. For instance, fungal hyphae have higher affinities for glutamate and higher capacities for glutamate uptake than mycorrhizal tree roots (Table 2). Fungi from the rhizosphere, including ectomycorrhizal fungi, may therefore be superior in the acquisition of amino nitrogen. This will be further discussed in Section 3.3.

Table 2
Kinetic parameters for uptake of glutamate by various organisms

Organism	V_{max} (μmol (g d. wt.)$^{-1}$ min^{-1})	K_m (μM)	Reference
Aspergillus nidulans	0.004	180	[77]
Paxillus involutus	0.293	27	[85]
Penicillium cyclopium	0.320	24	[77]
S. cerevisiae	–	30[a]–1000[b]	[75]
Spirodela polyrhiza	0.165	59	[148]
Hordeum vulgare	0.156	3	[149]
Eriophorum vaginatum[c]	0.010	118	[9]
Betula nana[c]*	0.012	70	[9]
Betula nana[d]*	0.012	176	[9]
Fagus sylvatica[e]*	0.029	113	(M. Chalot, unpublished)
Carpinus betulus[e]*	0.125	96	(M. Chalot, unpublished)
Aesculus hippocastaneum[e]*	0.054	253	(M. Chalot, unpublished)

[a]With system for acidic amino acids.
[b]With the general amino acid permease.
[c]From wet meadow community.
[d]From dry heath community.
[e]Mycorrhizas were sampled in mid-May and incubated for 2 h in [^{14}C]glutamate. Affinity constants and maximal velocities were determined according to Chalot et al. [85].
Mycorrhizal species are designated by an asterisk.

The inhibitory effect of external pH values higher than 6.4 on glutamate and glutamine uptake indicates a strong sensitivity of glutamate and glutamine uptake to the state of protonation, as already demonstrated for glutamate uptake by *Penicillium cyclopium* [77]. At pH values higher than 6, most of the glutamate and glutamine species carry a negative charge and are not transported. The optimal pH values for uptake of all amino acids studied in this work fall in the pH range normally found in forest soil [31]. Our data fit well with the co-transport theory, as already demonstrated for amino acid permeases from plants [79], yeasts and other fungi [77]. This theory is useful in that it provides a mechanism for coupling both proton gradients and membrane potential differences to the uptake of organic compounds. It also helps to explain why factors which alter the membrane potential or the proton concentration of the medium (protonophores, pH variation) may have significant effects on the uptake of substances which have a p*K* considerably above or below the pH of the medium.

The transport system characterized in *Paxillus involutus* seems to be rather unspecific. It was possible to inhibit the uptake of glutamate, aspartate, glutamine and alanine by various amino acids. The general amino acid transporter from *P. involutus* therefore resembles the amino acid permease of yeasts, which has more than 20 natural substrates, including most L- and D-amino acids, non-proteic amino acids and a number of toxic amino acid analogues or amino acid biosynthesis inhibitors [75,77,81]. The physiological significance of this transporter in symbiotic tissues will be discussed below.

3.2.2. Transport of peptides

The literature on peptide transport in fungi is limited to a few reports on *Neurospora crassa* and more extensive literature on the yeasts *Saccharomyces cerevisiae* and *Candida albicans* [86–88]. Small peptides (two to six amino acid residues) are transported across the plasma membrane as a source of amino acids and nitrogen [88]. This phenomenon is known to occur in a wide range of prokaryotes and eukaryotes including fungi and plants [87]. In all cases studied, peptide transport has been shown to be energy dependent and mediated by specific systems distinct from amino acid transporters. Information on molecular biology of peptide transport systems in eukaryotes is just emerging [87,88].

Fewer experiments have been performed with ectomycorrhizal fungi. Hexa-alanine was shown to be a less effective nitrogen source than shorter peptides for *Laccaria laccata*, *Suillus bovinus* and *Rhizopogon roseolus* [47]. Mycorrhizal birch plants were shown to be able to use N from peptides [89] and to further transfer the N to the shoot [90]. Such ability to utilize the N form peptides [91] and to further transfer their N to the host plant [42] was found with the ericoid endophyte *H. ericae*. However, most studies carried out so far on ectomycorrhizal fungi and ectomycorrhizas have used commercially available, but unlabelled, animal (BSA) or plant (gliadin) proteins or simple peptides (di-, tri- and tetra-alanine). No mixtures of simple ^{14}C or ^{15}N peptides and proteins are currently commercially available. Study of peptide and protein acquisition by ectomycorrhizal fungi and ectomycorrhizas requires a supply of peptides or proteins labelled in each amino acid residue and ideally derived from a range of different fungal or plant material but grouped according to molecular size [92]. Production of these types of compounds, which is under investigation in our laboratory according to a method developed by Ling et al. [92], including material derived from fungal tissue, will facilitate evaluation of the contribution of microbially derived organic compounds to the overall carbon and nitrogen economy of ectomycorrhizal forest trees. In preliminary experiments, we found that uptake of a mixture of [^{14}C]peptides by the ectomycorrhizal fungus *Paxillus involutus* was characterized by a lag phase during which the uptake of [^{14}C]peptides was low compared with uptake of amino acids. After 12 h of incubation, the uptake of [^{14}C]peptides was equal to that of amino acids (M. Chalot and J. Ling, unpublished). However, direct incorporation of peptides in fungal tissues remains to be demonstrated since utilization of peptides may arise as a result of exogenous peptidase activity. Utilization of peptidase inhibitors should allow to distinguish between exogenous versus internal degradation of oligopeptides. It is important to point out that the ability of yeast to transport and utilize peptides appears to be a non-inducible characteristic of *S. cerevisiae* [86]. These authors found no evidence for the induced synthesis of peptidases or peptide transport systems in the

strains they have used. The constitutivity of peptidases and peptide transport systems as well as the size limit for peptide transport remain to be established for ectomycorrhizal fungi.

3.2.3. Interaction between transport of different nitrogenous compounds

Neither NH_4^+ or NO_3^- had any effect on [^{14}C]amino acid uptake by the ectomycorrhizal fungus *P. involutus* when applied in the concentration range (0.5–0.05 mM) which could be expected in the forest soil solution [93], which suggests that uptake processes for organic and inorganic nitrogen sources operate quite independently [94]. This observation is of ecological interest because mixtures of both inorganic and organic nitrogen sources will normally occur in the natural environment [7,35]. These results are in good agreement with those of Kalisz et al. [95] who showed that the production of proteinases by basidiomycete fungi was not repressed by ammonium.

There is overwhelming evidence to show that amino acids and peptides are transported by different mechanisms. The most convincing support for the presence of a distinct transport mechanism is the lack of competition between peptides and amino acids during uptake. Experiments using [^{14}C]peptides deriving from plant tissues are in progress in our laboratory to investigate possible competition between peptide and amino acid transport in the ectomycorrhizal fungus *P. involutus*.

3.3. Transport of N by ectomycorrhizal tissues

Melin and Nilsson [10,11] first demonstrated long-distance transport of nitrogen compounds ([^{15}N]ammonium, [^{15}N]glutamic acid) from extramatricial hyphae of *Boletus variegatus* in mycorrhizal association with *Pinus sylvestris*. The role of ectomycorrhizal fungi in nutrient transfer is emphasized in mat communities formed in the upper soil profile by a number of ectomycorrhizal fungi. In these structures, ectomycorrhizal mats may act as nurseries for seedlings by transferring energy and possibly water and nutrients from mature host trees to Douglas fir seedlings [40]. In a later study [33], the authors concluded that the majority of the upward flow of nitrogen from the mineral soil to the litter layer observed in the forest soil was due to fungal translocation. However, the physiological processes involved in the transfer of nitrogen within the symbiotic tissues are still poorly understood. The exchange of nutrients between the fungus and the plant requires passage across the fungal cell wall and fungal plasmalemma and the interfacial matrix and plasmalemma of the plant. Consequently, the net transport of nutrient from the soil solution to the above-ground parts of the plant is the result of three transport components, one located at the soil-fungus interface and the two others located at the fungus-apoplast and apoplast-root cell interfaces. A number of hypotheses, which are derived from experiments on higher plants or bacteria, will be suggested below.

3.3.1. Transport of amino acids by mycorrhizal versus non-mycorrhizal roots

Experiments with axenic seedlings showed that non-mycorrhizal roots are able to take up amino acids, although at much lower rate than mycorrhizal roots [96,97]. In an attempt to better understand the physiological changes during fungal colonization of birch roots by *P. involutus*, we found for instance that the absorption rate of [^{14}C]glutamate by 15-day-old mycorrhizal roots was 10 times higher than that of non-mycorrhizal roots (D. Blaudez, M. Chalot, P. Dizengremel and B. Botton, unpublished). When compared with the absorption rates of the fungus in pure culture and when taking into account the infection degree of birch roots (by means of ergosterol content), it appeared that the higher capacity for glutamate uptake was solely due to the presence of the fungus. This is in contrast with previously reported results which demonstrated that the measured uptake rates for nitrate in mycorrhizal pine associated with *Hebeloma cylindrosporum* or *Pisolithus tinctorius* were greater than the calculated sum of the individual symbiotic partners [98,99]. However, choice of the fungal partner greatly affected uptake of amino acids by conifer species whereas choice of the host species had little effect on uptake rate [96,97]. For instance, rates of glycine uptake by roots of Douglas fir and western hemlock mycorrhizal with *Hebeloma cylindrosporum* and *Cenococcum geophilum* were respectively 25 and 33% higher than non-mycorrhizal seedlings, while rates

for roots mycorrhizal with *Suillus granulatus* were 75% lower than the control [96,97].

However, these experiments were carried out on axenic seedlings to preclude microbial degradation of the amino acids, and therefore the ecological significance of amino acid uptake by non-mycorrhizal roots is still unclear. Non-mycorrhizal *Pinus sylvestris* seedlings grown in unsterile soil conditions were able to take up as much organic nitrogen as the mycorrhizal plants [100]. The authors concluded that uptake of organic nitrogen by plants may be mediated by microorganisms other than ectomycorrhizal fungi, which has been suggested in a previous report showing the ability of the saprotroph *Oidiodendron griseum* to facilitate N transfer to the plants [49]. Conversely, Kielland [9] noted that ectomycorrhizal roots collected from forest soils had higher amino acid uptake capacities than did non-mycorrhizal roots. However, we found that ectomycorrhizal roots collected from forest soils had lower uptake capacities and lower affinity for glutamate than axenic mycelium (Table 2). The infrequent occurrence of non-mycorrhizal roots under natural conditions prevented us from comparing these results with those obtained with non-mycorrhizal roots. The nature of the transporters involved in the symbiotic associations is discussed further below.

3.3.2. The soil-fungus interface

In a study where we compared the uptake of [^{15}N]ammonium and [^{15}N]alanine by intact or disrupted ectomycorrhizal birch systems in perspex microcosm systems, we found that the uptake of both ammonium and alanine was significantly increased when birch seedlings were connected to an intact external mycelium (M. Chalot, K. Arnebrant, B. Söderström, unpublished). These results confirm previous findings that the external mycelium generally increases nutrient uptake and that it can be therefore considered as a nutrient channel. We can speculate that the amino acid transport system described for the pure culture fungus *P. involutus* [85] may also operate in the symbiotic association. The soil solution contains a mixture of amino acids [7,35] and therefore the presence of an amino acid transporter with low specificity but high uptake capacity is highly conceivable.

3.3.3. The fungus-apoplast interface

In the vast majority of symbioses the components of the interface are the opposed membranes of the two symbionts and the apoplastic region between them [13]. Changes in the distribution and activity of membrane-bound transport proteins in response to symbiotic interaction need to be investigated. According to Smith and Smith [13], the overall question to be considered in mutualistic symbioses is whether nutrient transfer processes have analogies elsewhere in the physiology of partners or whether new transport events are switched on as a result of interactions between the organisms.

The first intriguing question which arises when considering the transfer of amino acids at the fungus-plant interface is the nature of the mechanism involved at the fungus-matrix interface for export of nitrogenous compounds. Various hypotheses can be proposed, which are derived from studies on lower eucaryotes [72]. These hypotheses include:

1. an efflux of internally accumulated amino acids by diffusion. This hypothesis supposes that the amino acid transported must be sufficiently hydrophobic to give rapid efflux
2. a functional inversion of uptake systems. Carriers, like enzymes, in general catalyze reversible reactions. Since fungi possess amino acid uptake systems [85], a functional inversion under symbiotic conditions could be conceived
3. specific efflux carriers which have recently been described for bacteria [72].

Previous work has shown that the ectomycorrhizal fungus *Pisolithus tinctorius* is able to release amino acids into the external medium, and this efflux is greater when the fungus is incubated together with its host plant [99]. *Hebeloma cylindrosporum* could release amino acids when the colony was incubated in the presence of nitrate and the efflux could be modulated by externally applied glutamate [101]. We have recently examined the efflux of amino acids by *P. involutus* in pure culture. As demonstrated for amino acid uptake [68], efflux of the non-metabolizing amino acid analogue aminoisobutyric acid was highly dependent on the external pH, being minimal at acidic pH values (A. Brun, M. Chalot and B. Söderström, unpublished). However, the exact na-

ture of the efflux systems remains to be established. Smith and Smith [14] recently discussed the flux of phosphorus in arbuscular mycorrhizal symbiosis. They suggested the occurrence of a specific system such as an ion channel for the efflux of phosphorus from the fungus to the interface. Because the efflux of N compounds from the fungal cell to the apoplast probably concerns a limited range of amino acids (see below), a specific efflux system may be also involved at the fungus-apoplast interface for amino acid efflux. The underlying and interesting question is what induces the mycorrhizal fungus to give up its amino N, i.e. is there a signal involved in the specific efflux. This question cannot be answered until we have clear ideas of the mechanism(s) involved.

3.3.4. The apoplast-root cell interface

Although ectomycorrhizal tree roots are almost entirely covered with a fungal layer constituting a barrier between the soil component and the root cells, once they have been transported into the apoplastic region, amino acids have to be taken up by the root cells. The mechanisms involved in the uptake of amino acids from the apoplastic space by root cells may be inferred from experiments with herbaceous plants. The mechanism of amino acid uptake across the higher plant plasma membrane has been investigated in a number of plants, but mostly on shoot tissues where interest has centered on the regulation of phloem loading (for a review see [79]) and little work has been done on the uptake mechanisms by root tissues. Aspartic acid was shown to compete with nitrate and ammonium as a nitrogen source for pea suggesting the occurrence of specific transporters [102]. Roots of castor bean seedlings grown under natural or axenic conditions successfully compete with microorganisms for free amino acids in the soil [103]. In tundra ecosystems where plant uptake of organic nitrogen is observed [9], competition between plant and microbes is also suggested [8]. More recently, experiments on *Ricinus communis* seedling roots were consistent with transport of glutamine, glutamic acid and aspartic acid via a proton symport whereas transport of the basic amino acids, lysine and arginine, may be mediated by a voltage driven uniport [94,104]. A series of experiments carried out on the nitrogen nutrition of arctic plants, including tree species, unequivocally demonstrated that organic nitrogen may constitute a direct and unique nitrogen source thereby suggesting that these plants possess the required transport mechanisms [9].

The presence of an amino acid transporter with a high specificity may be expected at the apoplast root interface since not all amino acids are translocated at the interface. It remains to be established whether the nature of amino acid transport mechanisms involved in non-mycorrhizal roots differs from that of mycorrhizal roots or whether they share common properties. The nature of the amino acids transported is explored further below.

3.3.5. Nature of N compounds transferred

The nature of the N compounds transferred across the interface between symbionts has not yet been clearly proved but it can be inferred from labelling experiments as discussed by Smith and Smith [13]. The result of organic N or ammonium N assimilation in mycorrhizal roots will be a mixture of glutamine and glutamate and further products thereof (alanine, aspartate, γ-aminobutyric acid, citrulline). Movement of nitrogen has been studied in microcosms containing pine, alder or beech seedlings infected with the ectomycorrhizal fungus *Paxillus involutus* [24,25,105]. Inorganic nitrogen fed to an external mycelium has been shown to be rapidly incorporated into free amino acid pools and further translocated to the infected plants. In pine seedlings, the free amino acids with the highest ^{15}N enrichment levels, as well as ^{15}N concentration, were glutamine, asparagine and citrulline. We can therefore assume that these compounds are probably good candidates for translocation between the fungal cells and the root cortical cells since they are also the major component of the N pool in the xylem of tree plants [2]. Similarly, when mycorrhizal birch plants infected with *Paxillus involutus* were incubated in ^{15}N-labelled alanine for short periods, [^{15}N]glutamate and [^{15}N]glutamine but also [^{15}N]alanine were found in greatest amounts in shoots [106]. Interestingly, when plants were fed with aminooxyacetate, an inhibitor of alanine aminotransferase, only [^{15}N]alanine was found in shoots, suggesting that alanine could also be transferred.

3.4. An overview of transport systems for nitrogenous compounds in mycorrhizas

A hypothetical scheme for absorption of nitrogenous compounds is given in Fig. 2. Extracellular digestion of proteins is carried out by proteases as discussed in Section 2. The resultant end products, mostly large peptides, undergo further hydrolysis by peptidases excreted by the fungus. Small peptides, primarily those consisting of two or five amino acids, are transported intact across the plasma membrane via specific peptide transport systems. Fungal cells may therefore contain cytosolic peptidases that rapidly hydrolyze these peptides, generating free amino acids. Free amino acids are also absorbed into the fungal cell across the plasma membrane via amino acid specific transport systems, probably of low specificity. The internal amino acid pool may also be fed by amino acids resulting from the assimilation of inorganic nitrogen which mainly occurs in fungal cells. The overall process of nitrogen uptake therefore leads to the production of intracellular amino acids which will be further transferred to the aerial parts of the host plant.

The nitrogenous compounds entering into the export pathway are mostly amino acids. Therefore if the study of transporters for inorganic nitrogen compounds and peptides is limited to the soil-fungus interface, the study of amino acid transport systems appears far more complicated with the occurrence of transporters with various specificities located at different interfaces of the symbiosis. It is expected that this particular research area will need powerful tools to answer specific questions which arise from the considerations above. Indeed, nitrogen transport processes in fungi and higher plants are poorly understood, mainly because it is difficult to purify integral membrane proteins and to assay their activ-

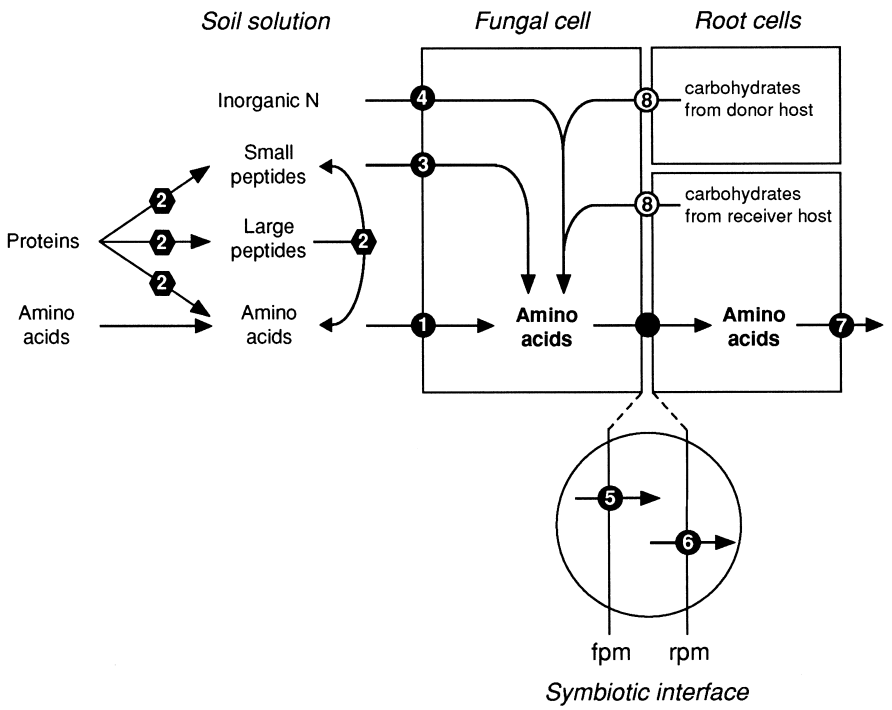

Fig. 2. Hypothetical model for uptake and transfer of nitrogenous compounds through the soil-fungus and fungus-plant interfaces. (1) Fungal membrane amino acid transporter(s) of low specificity, (2) proteolytic activities for exocellular hydrolysis of proteins and peptides, (3) fungal membrane oligopeptide transporter, (4) fungal membrane transporters for ammonium or nitrate, (5) fungal membrane efflux system for amino acids, (6) and (7) plant membrane amino acid transporter(s) of high specificity, (8) sugar transporter(s). fpm: fungal plasma membrane, rpm: root plasma membrane.

ity. The application of modern molecular techniques to the study of nitrogenous compound transporters in ectomycorrhizal fungi will, no doubt, lead to a better understanding of many aspects of amino acid and peptide transport that are not well defined at present. Genes encoding phosphate [107] and sugar (U. Nehls, A. Wiese and R. Hampp, personal communication) transporters have been recently cloned in the mycorrhizal fungus *Glomus versiforme* and spruce roots, respectively, and there is an urgent need for similar molecular characterization of nitrogenous compound transporters in different mycorrhizal associations.

The scheme also includes the recent view (carbohydrate from donor host) that amino acids may served as transfer compounds between two host trees connected by a common mycelium [108]. This will be further discussed in Section 4.2.

4. Intracellular degradation of amino acids

Plant, animal and microorganism residues as well as root exudates are the main sources of amino acids in soil [109–113]. Fogel [38] estimated that mycorrhiza accounted for 43% of the N released annually in a Douglas fir ecosystem, followed by fungi (35%) and litter (14%). Actinomycetes isolated from soil-free, rhizosphere, and mycorrhizosphere of pine produced glutamate and alanine in greatest amounts [110]. Presumably these compounds may serve as carbon and nitrogen sources for mycorrhizal fungi. In addition to being a readily available source of carbon and nitrogen, amino acids are the immediate products of ammonium assimilation by ectomycorrhizal fungi. A number of recent reviews have focused on the enzymology of ammonium assimilation by ectomycorrhizal fungi [19,20] and this will therefore not be dealt with in detail in this review. In short, most of the results available to date suggest that the main pathway for ammonium assimilation is via the glutamine synthetase/glutamate synthase cycle [114–117] whereas the glutamate dehydrogenase pathway plays a minor or no role. This alternative pathway has been shown to be operative under conditions where ammonium assimilation through GS is inhibited [118] but its contribution is difficult to assess because of the absence of specific inhibitors. This chapter will focus on the acquisition of amino acids as carbon and nitrogen sources.

Table 3
Growth (mg dry weight) of ectomycorrhizal isolates on various organic nitrogen sources

	Ala	Arg	Asn	BSA	Glu	Gln	Gly	Lys	Ref.
Amanita sp.	–	38	126	47	–	112	–	–	[7]
Gautieria sp.	–	12	8	12	–	88	–	–	[7]
Elaphomyces sp.	–	128	106	13	–	134	–	–	[7]
Pisolithus sp.	–	6	74	9	–	103	–	–	[7]
S. bovinus	103	109	94	–	104	96	19	55	[118]
A. muscaria	83	131	90	–	140	72	87	69	[118]
H. crustuliniforme	81	62	75	–	98	72	39	23	[118]
P. involutus	37	137	–	106	81	–	–	–	[120]
T. terrestris	84	74	121	16	12	–	–	–	[120]
H. crustuliniforme	93	93	47	53	86	–	–	–	[120]
P. involutus	–	–	23	5	–	–	7	–	[121]
A. muscaria	–	–	85	11	–	–	69	–	[121]
L. rufus	–	–	68	<1	–	–	4	–	[121]
P. grandis microbiont	–	227	–	217	200	–	–	–	[123]

Growth is expressed as a percentage of that on ammonium.
[7]: growth for 28 days, pH 5.5.
[118]: growth for 30 days, pH 5.0.
[120]: growth for 60 days, pH 5.5 calculated from figures.
[121]: growth for 30 days for *P. involutus* (strain Pa 1-2), 40 days for *L. rufus* (strain La 5-1), 60 days for *A. muscaria* (strain Am 3-18), pH 5.5.
[123]: growth for 21 days, pH 5.5 calculated from figures. Unidentified mycobiont from *Pisonia grandis*.

4.1. Amino acids as nitrogen sources

That ectomycorrhizal fungi are able to utilize amino acids for growth has been demonstrated repeatedly [117,119–122]. Indeed, most of the ectomycorrhizal species investigated so far are able to grow on amino acids as a nitrogen source (Table 3). Ericoid endophytes were less selective in their utilization of amino acids than are ectomycorrhizal fungi [123]. The unidentified mycobiont isolated from mycorrhizas of *Pisonia grandis* was able to grow on a variety of organic nitrogen sources (including allantoin, urea, and BSA) at higher rates than on inorganic nitrogen sources [124]. However, Table 3 also shows that there are differences in the ability of ectomycorrhizal fungi to utilize single amino acids, the amido-nitrogen of amino acids being more suitable than the amino-nitrogen [62,122]. Indeed, glutamine and asparagine are often readily utilized by most of the fungi examined whereas glycine is less usable. In contrast, glutamate and alanine often support growth similar to that on ammonium for most isolates (Table 3). A high degree of heterogeneity is also found between isolates within the same species for growth on a single amino acid [62,121,122]. These observations have ecological implications in terms of succession and forest development, however the establishment of further correlations between fungal type, vegetation and soil characteristics is necessary for these to be properly assessed.

Glutamate, glutamine and alanine, which showed less variation in terms of growth of most ectomycorrhizal isolates tested, are also the main amino acids from the free pools in ectomycorrhizal fungi and ectomycorrhizas and have therefore been used extensively in growth experiments [47,48,121] and for a better understanding of the metabolic pathways involved in their degradation [115,125,126]. In free-living mycorrhizal mycelia and ectomycorrhizas, after a primary incorporation of externally supplied $^{15}NH_4^+$ into glutamate and glutamine, the N of glutamate and glutamine is incorporated into a range of amino acids, mainly alanine, aspartate and asparagine after short [23,26] or long [24,25] incubation periods. These findings, supported by the high aminotransferase activities measured in ectomycorrhizas and ectomycorrhizal fungi [127–129], stress the central role of glutamate and glutamine as N donors. Melin and Nilsson [11] suggested that glutamic acid provided to the fungal partner might have been transaminated in the hyphal cells before being transferred to the host. Conversely, isotopic investigations using ^{15}N-labelled alanine as a substrate have clearly shown that *P. involutus* utilizes the alanine aminotransferase pathway to assimilate alanine, both in pure culture [126] and in symbiosis [106]. Not surprisingly, glutamate, glutamine and aspartate were shown to be the main sinks for the amino group derived from alanine.

The importance of glutamine as an essential intermediate in N metabolism and/or as a storage N compound in ectomycorrhizal fungi makes the study of this amino acid particularly necessary. For instance, the degradation of glutamine by the possible glutamine transaminase/ω-amidase sequence as an alternative pathway to GOGAT, as demonstrated for *Neurospora crassa* [130], is currently under investigation in our laboratory by using ^{15}N-amido- and ^{15}N-amino-labelled glutamine.

4.2. Amino acids as carbon sources

Heterotrophic carbon assimilation by mycorrhizal plants of birch has been estimated using ^{14}C-labelled proteins [131]. Assuming that breakdown products of proteins are assimilated as amino acids, the authors calculated that 9% of plant C may be derived from protein. However, the metabolic pathways for the acquisition of C derived from amino acids were not investigated in that study. More recently, data on the ectomycorrhizal fungus *P. involutus* have provided direct evidence for the utilization of glutamate and glutamine carbons via glutamine synthetase and glutamate synthase, which are further actively channelled through the TCA cycle, thus providing a carbon source for mycelial respiration and for amino acid biosynthesis through transamination reactions [115]. The pivotal role of aminotransferases in the conversion of amino acids was further stressed by the utilization of [^{14}C]alanine. When *P. involutus* was fed with alanine together with the aminotransferase inhibitor aminooxyacetate, alanine was not metabolized but rather accumulated in the mycelium [125]. The high recovery of label from amino acids

other than alanine suggested that amino acid synthesis is an important sink for TCA cycle intermediates. This is in good agreement with ^{13}C nuclear magnetic resonance experiments showing that formation of glutamate and glutamine is a major sink of TCA cycle intermediates in [^{13}C]glucose-fed *Cenococcum geophilum* [132].

Conclusions from studies of carbon uptake and metabolism by isolated mycelium need to be confirmed with mycorrhizal fungi in their natural environment, i.e. in the intact mycorrhizal system. There have been numerous studies that have compared the respiration rates of freshly excised ectomycorrhizal roots with those of non-mycorrhizal roots. These studies generally show that mycorrhizal roots have greater respiration rates per gram dry weight than non-mycorrhizal roots (for reviews see [3,99]). Söderström and Read [133] demonstrated that approximately 30% of the total respiration in intact ectomycorrhizal mycelial systems was attributable to the mycorrhizal mycelium in the soil. It was recently demonstrated that the mycorrhizal symbiont *Hebeloma crustuliniforme* increased below-ground respiration, thus decreasing overall retention of carbon within the plant-fungus symbiosis [134]. Recent experiments using [^{14}C]alanine showed that *Paxillus involutus* doubled the amount of label taken up by the birch root system [106]. However, the total amount retained within the roots was the same irrespective of mycorrhizal status (Fig. 3). This was because 60% of the label was lost in the form of respired CO_2 from mycorrhizal root systems, compared with only 28% in non-mycorrhizal plants. These results with intact mycorrhizal systems confirm our earlier results with isolated mycelium, which showed that a significant proportion of provided labelled substrate, such as alanine, is actively respired.

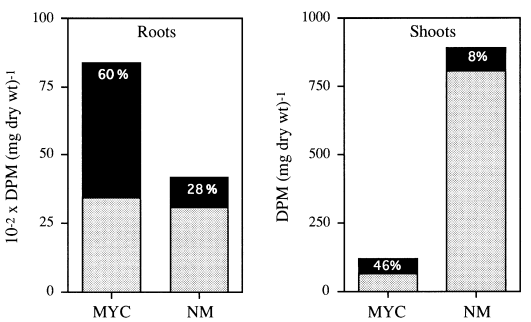

Fig. 3. Uptake and respiration of [^{14}C]alanine by ectomycorrhizal (MYC) and non-mycorrhizal (NM) birch seedlings. Plants were fed with [^{14}C]alanine for 2 h. At the end of the uptake period, roots were washed for 5 min with 0.1 mM $CaSO_4$ prior to radioactivity counting. Radioactivity in tissues (hatched bars) was measured by scintillation counting after tissue solubilization. Radioactivity evolved as $^{14}CO_2$ (black bars) was measured after trapping the CO_2 in a mixture of methanol/ethanolamine (70/30, v/v).

These considerations are important in terms of C translocation between trees interconnected by a common mycelial network. Recent studies have quantified the extent of interspecific C transfer between paper birch and Douglas fir and have unequivocally demonstrated net C transfer from paper birch to Douglas fir [108,135]. Although the role of the ectomycorrhizal mycelium remained uncertain to the author, it was suggested that C could be translocated between the two plants along a nitrogen concentration gradient in the form of amino acids. The role of ectomycorrhizal partners is therefore expected to be of central importance in metabolizing C compounds from a donor host to C-containing nitrogenous compounds transferred to a receiver host (Fig. 2). This is an extension of the common view that in ectomycorrhizas mutual benefit between the two partners results from the exchange of host-derived carbohydrates for amino acids supplied by the fungus [3,136].

4.3. Nitrogen-carbon interactions in ectomycorrhizal fungi and ectomycorrhizas

The interactions of nitrogen and carbon in the physiology of ectomycorrhizal fungi and ectomycorrhizas has been extensively reviewed (for reviews see [99,137]). This was further supported by recent experiments which demonstrated that when *Paxillus involutus* either symbiotic or non-symbiotic was fed with the organic acid [^{14}C]malate, most of the radioactivity was recovered in the amino acid fraction (D. Blaudez, M. Chalot, P. Dizengremel and B. Botton, unpublished). Conversely, recent results showing that amino acids were intensely respired [115,125] support the conclusion that amino acids may serve not only as nitrogen sources but also as carbon sources for ectomycorrhizal fungi and ectomycorrhizas. The studies of key enzymes such as the aminotransferases

[128,129] or more recently the isocitrate dehydrogenases [138] which provide keto acid skeletons for ammonium assimilation are important for understanding the co-regulatory processes of both nitrogen and carbon metabolisms in fungal and symbiotic tissues.

It is evident, from studies of ectomycorrhizal development, that structural and functional integration of the root and fungus can take place very rapidly [139–141]. Therefore, future research will need to address the molecular and biochemical regulation of enzymes involved in nitrogen and carbon metabolisms during early mycorrhiza formation.

5. Immunogold localization of amino acids in fungal cells

As demonstrated above, amino acids are of crucial importance in nitrogen nutrition of ectomycorrhizal fungi and ectomycorrhizas, not only as metabolic intermediates and building blocks of proteins, but also as storage compounds. This creates different pools within the cells which may have different functions. We have recently suggested that a very small but metabolically active pool of glutamate, serving as a substrate in glutamine synthesis may be possibly tightly compartmentalized, away from the other glu-

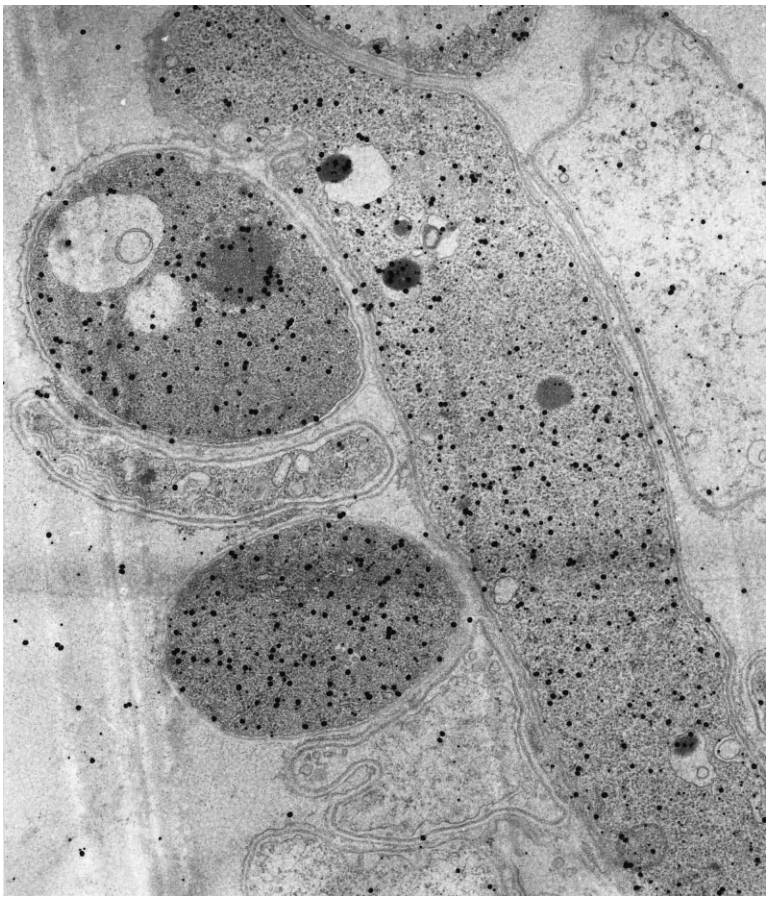

Fig. 4. Immunogold localization of glutamine and glutamate in *Paxillus involutus*. Free living mycelium of *Paxillus involutus* was taken up from the actively growing edge of the colony, fixed with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde, postfixed with 1% osmium tetroxide and embedded in an epoxy resin (Epon 812) and polymerized at 60°C for 48 h. Ultrathin sections were mounted on nickel grids and processed for cytochemical labelling. Double labelling with anti-glutamate (10 nm gold particles) and anti-glutamine (30 nm gold particles) sera was performed by running two sequential incubations (anti glutamate first), according to [145]. 1 cm represents 0.55 μM.

tamate pool which channels the carbon flow from catabolism of glutamine and serves as a source of carbon skeletons in the synthesis of organic acids and amino acids [115]. This is consistent with a previous work showing clear compartmentation of glutamine synthetase in the ectomycorrhizal fungus *Laccaria laccata* [142]. This dual role of amino acids calls for a new approach when attempts are made to analyze the distribution of amino acids in fungal cells. Electron microscopy combined with immunogold labelling is a method of choice to localize cellular components and it has been widely applied for localization of proteins. This technique however relies on the availability of antisera, which are easily produced from purified proteins. A number of antisera have been produced during the past few years for amino acid metabolizing enzymes such as aspartate aminotransferase [128], NADP-glutamate dehydrogenase and glutamine synthetase [143] purified from ectomycorrhizal fungi and further used for immunogold localization of enzymes in tissues [142,144]. Our interest has recently focussed on the possibility of using antisera produced again amino acids to localize them in fungal and symbiotic tissues. Recent methodological developments have made it possible to utilize specific antisera to explore the cellular and tissular distribution of amino acids. Antibodies raised again glutamate, glutamine and arginine have been produced by Storm-Mathisen and his collaborators [145], which allow the use of immunogold labelling techniques.

The localization of amino acids was investigated in cells of the ectomycorrhizal fungus *Paxillus involutus* (Batsch) Fr. by electron microscopy of ultrathin stained with gold labelled antibodies. Immunogold labelling of fungal cells clearly showed a cytosolic localization of glutamate and glutamine. When using a double labelling protocol, with two particle sizes (10 and 30 nm) for glutamate and glutamate, respectively, it appeared that glutamine amount was 3–5-fold higher than glutamate amount (Fig. 4) which confirmed quantitative analyses. However, there was a great heterogeneity of labelling distribution between different cells, with cells intensely labelled whereas other cells were poorly labelled, which is not reflected by extraction of amino acids from whole tissues and further analysis by chromatography. In particular, the presence of electron dense vacuolar bodies with high labelling for glutamate and glutamine confirmed the occurrence of compartmentation of amino acids in fungal cells (Fig. 4). Nitrogen containing vacuolar bodies were also visualized in the hyphal sheaths of *Xerocomus badius-Picea abies* ectomycorrhizas [146], and were further shown to contain high levels of amino acids (A. Brun and I. Kottke, unpublished). Using microautoradiography, Sangwanit [97] showed that [^{14}C]glycine was stored in the mantle/Hartig net of mycorrhizal roots, while nonmycorrhizal roots rapidly moved the glycine into the stele and hence the shoots.

This study represents a step towards the use of electron microscopy immunocytochemistry as a tool to assess absolute concentrations of amino acids in fungal cells and symbiotic tissues. The availability of antibodies against the essential amino acid glutamine will also allow us to investigate the possible role of this amino acid in transfer mechanisms at the symbiotic interface.

6. Perspectives

As pointed out by Alexander [39], ectomycorrhizas are found in the nitrogen-rich organic layers of humic soil horizons and there is no doubt that they actively participate in the degradation of organic bound N. This review aimed to emphasize the possible physiological mechanisms underlying the role of ectomycorrhizal partners in the mobilization of organic nitrogen. In spite of the recent gains made in the field, much remains to be understood in critical areas, especially those dealing with the molecular nature, and regulation of transport systems and metabolic pathways.

The assumptions that the properties of fungal plasma membrane are somehow related to the ecological role of fungi [70,71] highlight the need for exploring the role, the distribution and the structure of transport proteins in ectomycorrhizal fungi and ectomycorrhizas. Efforts to clone transport system genes have met with considerable success in recent years in plants. The method of choice for cloning transporter genes from eukaryotes relies on the complementation of yeast mutants [147]. This approach

will greatly expand our knowledge on the molecular nature of these transport systems in ectomycorrhizal fungi and ectomycorrhizas. An intriguing question for which there is currently no answer is how these transport systems are differentially sorted in the Hartig net cells. Development of transport-system-specific antibodies raised against peptide sequences deduced from cDNAs or against purified transport proteins will be of enormous help in answering this question. The cDNA and the antibody probes will also assist in unravelling the mechanisms involved in the regulation of these transport systems, both at the genetic level and at the protein level. This approach currently under investigation in our laboratory will be directed to elucidate nitrogen transport mechanisms at the biotrophic interface during the early stages of mycorrhiza formation and will follow changes in the distribution and activity of membrane-bound transport proteins in response to symbiotic interactions.

Recently, a gene encoding glutamate dehydrogenase has been cloned and sequenced in the ectomycorrhizal fungus *Laccaria bicolor* (Lorillou and Martin, personal communication) and we expect a number of genes encoding other essential enzymes to be cloned and characterized in the near future. Further site-specific mutagenesis of fungal cloned DNA fragments should allow us to analyze gene functions as well as to explore enzyme structure and function and their regulation under symbiotic or non-symbiotic situations.

An area in this research field that needs future expansion is the study of uptake of nitrogenous compounds by tree roots from different communities in their field situation. Several fungal species commonly occur on a single root tip, and many more can inhabit the root system of a single host plant [12]. Moreover, competition for nutrients may occur not only between different fungal species, but also between genetically different individuals of the same species or between different microbial communities, including saprotrophs and bacteria [54], thus adding to the complexity of the overall process of nitrogenous compound acquisition. Therefore more research is needed to understand variation from the species to the community level and to correlate the behavior of a single ectomycorrhizal isolate to its environment.

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