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Recent advances in exploring physiology and biodiversity of ectomycorrhizas highlight the functioning of these symbioses in ecosystems

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Abstract

Ectomycorrhizas, the dominating mycorrhizal symbiosis in boreal, temperate and some tropical forests, are formed by 5000–6000 species of the asco- and basidiomycetes. This high diversity of fungal partners allows optimal foraging and mobilisation of various nitrogen and phosphorus forms from organic soil layers. In this review, two approaches to study the functioning of this multitude of symbiotic associations are presented. On selected culture models, physiological and molecular investigations have shown that the supply of hexoses has a key function in controlling the plant–fungus interaction via partner-specific regulation of gene expression. Environmental factors which affect fungal carbon supply, such as increased nitrogen availability, also affect mycorrhiza formation. Based on such laboratory results, the adaptative capability of ectomycorrhizas to changing field conditions is discussed. The second approach consists of analysing the distribution of mycorrhizas in ecosystem compartments and to relate distribution patterns to variations of ecological factors. Recent advances in identification of fungal partners in ectomycorrhizas by analysing the internal transcribed spacer of ribosomal DNA are presented, which can help to resolve sampling problems in field studies. The limits of the laboratory and the field approaches are discussed. Despite some problems, this combined approach is the most promising. Direct investigation of gene expression, which has been introduced for soil bacteria, will be difficult in the case of mycorrhizal fungi which constitute organisms with functionally varying structures.

Keywords: Ammonium assimilation; Carbon allocation; Ectomycorrhiza; Gene expression; Sugar transport; rDNA; Internal transcribed spacer; Molecular marker; Ectomycorrhizal community; *Amanita muscaria*; *Picea abies*

Contents

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1. Introduction

The term 'mycorrhiza' describes the symbiotic association of plant roots with fungal hyphae. About 90% of the terrestrial plants belong to families that are commonly mycorrhizal [79] and mycorrhizas are considered as 'the chief organs involved in nutrient uptake of most land plants' [50,103,107]. As plants can manage without fungal partners, for example on soils without nutrient and water limitation or in well fertilised fields, mycorrhizas are not a strictly obligate symbiosis. However, in most terrestrial habitats, they have a crucial role for development and stability of plant communities and are therefore regarded as 'ecologically obligate' [96]. From the point of view of their role in ecosystems, three major forms of mycorrhizas are recognised: the ericoid mycorrhizas, the arbuscular mycorrhizas and the ectomycorrhizas (ECM) [62]. Only ECM will be dealt with in this overview (for an exhaustive review of mycorrhizal types and of their respective organisation, see Smith and Read, [108]).

ECM establish with the fine roots of autotrophic trees and shrubs, especially of the families *Betulaceae*, *Pinaceae*, *Fagaceae*, *Salicaceae* and *Dipterocarpaceae* [95,108]. The fungal partners belong to the basidiomycetes and ascomycetes. Typically, hyphae form a mantle of varying thickness around the fine roots. From there, hyphae or more specialised hyphal aggregates (rhizomorphs) radiate into the substrate in order to exploit nutrients and water. Mantle hyphae also extend into the apoplast of the root cortex. Here they form highly branched networks which establish a large surface area for solute exchange. This structure is called the Hartig net and constitutes the interface for the exchange of photoassimilates, soil water and nutrients between the host plant and its fungal partner.

Communities of ECM trees dominate in the boreal and temperate plant biomes and are important also in certain tropical rain forest environments [96]. In these diverse plant formations, ECM fungi are the best adapted to mobilise the sparse heterogeneous resources in phosphorus and especially in nitrogen from the litter layer. This function is ensured by a high diversity of fungi, which has been estimated between 5000 and 6000 species [79]. This high biodiversity of ECM fungi corresponds to a broad range of capabilities for the uptake of specific forms of organic and inorganic nitrogen and phosphorus, allowing the development of tree vegetations with a low plant species diversity despite the above mentioned heterogeneity and limitation of nutrient resources [96].

In this context, specific questions are addressed concerning the formation and regulation of ECM symbioses. Besides the role of photoassimilate supply, the question of the nitrogen supply and of the role of the diversity of the fungal partners are of crucial importance. More than in other mycorrhiza types, such as arbuscular or ericoid mycorrhizas which involve less diversity in fungal partners [108], a dual approach combining laboratory studies on selected model associations and field investigations is necessary in research on ECM. This review addresses the importance of this dual approach. It first summarises the actual knowledge about the plant–fungus interaction in ECM on the basis of carbon and nitrogen availability as investigated on selected laboratory models. This approach is the most promising for elucidating the key mechanisms that control establishment and regulation of the symbiosis. Second, it presents the most recent advances in the effort to investigate ECM in the field, underlining progress toward the analysis of structural diversity with molecular tools. Relating species and population structures to variations of ecological factors should provide a more comprehensive view of the function and role of ECM in ecosystems. However, as illustrated by a few examples, attempts to analyse functional diversity in this way only allow limited advances toward a causal analysis of functional diversity in ECM. This is due to technical difficulties as well as to the size of fungal organisms.

2. The carbon/nitrogen balance in ECM: lessons from investigations with model systems

2.1. The role of carbon for mycorrhiza formation

2.1.1. Fungal needs

Only a relatively small number of ectomycorrhizal fungi can be maintained in pure culture on synthetic media. They can all grow on a single organic carbon source, which is probably a property common to all ECM fungi. In symbiosis, organic compounds contained in root exudates are of special interest as putative candidates for carbon transfer from the host to the mycorrhizal fungus. Low-molecular-mass root exudates comprise soluble sugars, carboxylic acids and amino acids ([72]; for recent overviews, see [46,108]).

Cell wall precursors exuded by the plant cells have been discussed as an additional carbon source for mycorrhizal fungi [50,102,106]. Owing to the rates of carbon consump-

tion by the fungal partner, which is up to 30% of the net photosynthesis rate [48,108,109], this is, however, not likely to constitute the major carbon source.

Taken together, there is evidence that mycorrhizal fungi can use sources other than the 'classical' hexoses, but the significance of these alternatives is unclear and needs further experimentation.

2.1.2. Host supply

One of the first attempts to assay carbon flow in a mycorrhizal plant was performed by Melin and Nilsson [77]. They showed that after feeding ^{14}C-labelled CO_2 to leaves, labelled carbon appeared within 1 day in the hyphal mantle.

In general, the direction of carbon flow in the host is controlled by gradients between production (source organs such as leaves) and consumption (sink organs such as non-green tissues) of photoassimilates. Carbon will thus always be directed to the most active sink area in a plant, and the sink strength has been shown to control the rate of photoassimilate production [93,111]. This regulatory mechanism should be especially effective in mycorrhizal roots where the fungal partner constitutes a very strong sink. Indeed, there is ample evidence that mycorrhization directs assimilates toward the root and can up-regulate the rate of net photosynthesis of the host ([27,71,86]; see also [108]).

One of the key regulatory steps is probably that of sucrose synthesis in the leaf cytosol. This is mainly via fructose bisphosphatase and sucrose phosphate synthase. Fructose bisphosphatase activity is inhibited by an effector metabolite, fructose 2,6-bisphosphate (for reviews, see [93,110]). For spruce seedlings, it was shown that the amount of this regulator was greatly decreased in source needles of mycorrhizal versus non-mycorrhizal plants [48,71]. Sucrose phosphate synthase catalyses a reaction which is regarded as essentially irreversible in vivo [112]. The enzyme is subject to regulation by metabolites (glucose 6-phosphate activates and inorganic phosphate inhibits; [26,104]), and by protein phosphorylation [56,70], while regulation by protein synthesis/degradation does not appear to be of significance in the short term. Phosphorylation of sucrose phosphate synthase results in deactivation, i.e. lower sensitivity toward the activator, but increased sensitivity to the inhibitor. The properties of sucrose phosphate synthase respond to mycorrhization. For spruce seedlings, it could be shown that the phosphorylation of sucrose phosphate synthase is lower in mycorrhizal than in non-mycorrhizal source needles [70,71]. In addition, the affinity of this enzyme for fructose 6-phosphate was increased by mycorrhization in both spruce and aspen [71]. Both observations, decreased levels of fructose 2,6-biphosphate and increased activation of sucrose phosphate synthase, are thus indicative for an increased capacity for sucrose formation in mycorrhizal plants which is in agreement with the observation of increased rates of net photosynthesis (see above).

The driving force for sucrose allocation is assimilate consumption at the sink site. In ECM, this is obviously influenced by the fungus. Generally, tracer studies indicate rapid translocation of ^{14}C-labelled assimilates to the roots of ectomycorrhizal plants [22,49,77,106], especially in young symbiotic interactions [15].

An attempt to assay the longitudinal distribution of soluble carbohydrates within mycorrhizal roots was made by Rieger et al. [99]. Sucrose, but not glucose or fructose concentrations varied longitudinally. Sucrose levels were lowest in the middle parts of a mycorrhiza, i.e. the location of most intense symbiotic interaction. Correspondingly, levels of fungus-specific compounds such as trehalose or ergosterol were increased in this area [47]. Fine roots without fungal infection, in contrast, did not show longitudinal variations in sugar content.

2.2. Solute exchange at the apoplastic interface

2.2.1. Exchanged solutes

Experiments with suspension-cultured hyphae of *Amanita muscaria* and *Hebeloma crustuliniforme* [101] and with protoplasts of *A. muscaria* [20] indicated that these ectomycorrhizal fungi take up glucose and fructose, but have no system for sucrose transport. There is evidence that sucrose can be used as a carbon source by these ectomycorrhizal fungi only if it is hydrolysed by the cell wall-bound invertase of their host [101].

In addition to invertases, roots contain sucrose synthase, another sucrose-splitting enzyme. A histochemical analysis showed for spruce that cell wall-bound acid invertase dominates in the root cortex, the area of symbiotic interaction, while minor activities of sucrose synthase could be detected in the vascular bundle only [102].

Up to date, no clear evidence for the presence of sucrolytic enzymes in ectomycorrhizal fungi has been provided.

2.2.2. Hexoses/fungal transport systems

Transport studies with protoplasts isolated from *A. muscaria* and *Cenococcum geophilum* showed that these exhibited a preference for glucose over fructose while sucrose was not taken up [20,47].

Using a molecular approach, a monosaccharide uptake system was identified in *A. muscaria* and termed AmMST1 [82]. The function of the AmMST1 protein as an active monosaccharide transporter was confirmed by heterologous expression of the full length cDNA in a yeast mutant, lacking a functional endogenous monosaccharide uptake system ([122]; Fig. 1). The uptake properties of the AmMST1 monosaccharide transporter resemble those obtained with *A. muscaria* protoplasts [20,121]. The K_M value for glucose was determined as 0.4 mM, that for fructose as 4 mM. Also, a strong inhibition of fructose uptake in the presence of glucose was found.

A. muscaria hyphae grown at glucose concentrations below 5 mM expressed the AmMST1 gene at a basal level,

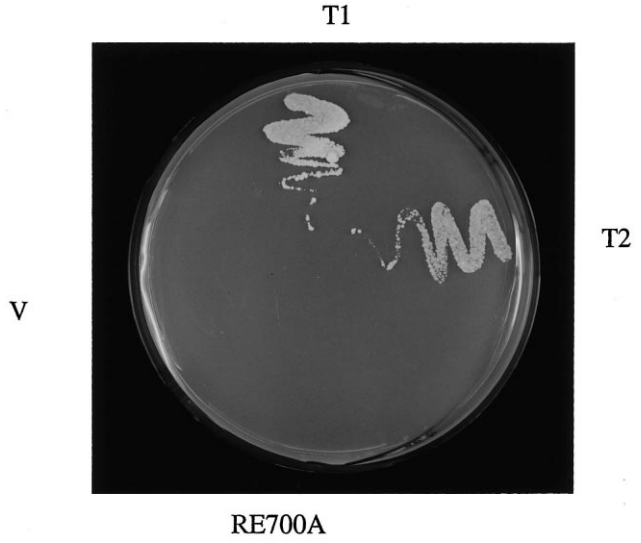

Fig. 1. Growth of *S. cerevisiae* RE700A transformed with a construct expressing *AmMST1* and the vector without insert. (T1 and T2) independent transformants containing the *AmMST1* expression vector, (V) yeast transformant containing the expression vector without insert, (RE700A) untransformed yeast mutants were grown on agar plates containing 2% glucose as a sole carbon source.

while monosaccharide concentrations above this threshold caused a 4-fold increase of the amount of the AmMST1 transcript. An increase of AmMST1 expression, similar to that found in fungal mycelia when cultivated at elevated monosaccharide concentrations, was also observed in symbiosis of *A. muscaria* with both the gymnosperm *Picea abies* and the angiosperm *Populus tremula*×*tremuloides*. It can thus be assumed, that the increase of AmMST1 expression in mycorrhizas is also sugar-regulated and depends on the in vivo concentration of monosaccharides at the fungus/plant interface.

Interestingly, at the threshold value of 5 mM glucose, the uptake capacity of the *Amanita* monosaccharide transporter (see above for investigations with fungal protoplasts) is nearly saturated. Apparently, the fungus can sense saturation of monosaccharide transport and in due course enhances AmMST1 expression. This results in additional transporter proteins with a concomitant increase of the monosaccharide import capacity, obviously an adaptation to the conditions during symbiotic interaction. The enhanced photoassimilate sequestration by the fungus should then trigger photoassimilate supply by the host with the consequence of increased rates of photosynthesis (see above).

The increase of AmMST1 expression is a slow process. A transition from constitutive to maximal content of AmMST1 transcripts occurred between 18 and 24 h of fungal culture. This is possibly due to the fact that mycelia growing in the soil are exposed to low concentrations of carbohydrates [118]. Higher monosaccharide concentrations were found only locally and for limited times.

In contrast to hyphae growing in the soil, those hyphae at the symbiotic interface are exposed to a continuous supply of plant-derived carbohydrates (see above). In order to adapt the flux of carbohydrates into the fungus, monosaccharides must be quickly taken up and either metabolised or converted into compounds for intermediate or long term storage (e.g. trehalose, mannitol, glycogen). It can thus be assumed that both the extended lag phase for enhanced AmMST1 expression and its threshold response to elevated monosaccharide concentrations are adaptations of the ectomycorrhizal fungus to the conditions found at the symbiotic interface.

2.2.3. Hexoses/host transport systems

In contrast to the fungal partner, monosaccharide transport in spruce root cells is not increased upon mycorrhization [81]. Expression of the respective gene (only one putative hexose transporter could be identified by Southern analysis) appeared even to be decreased under conditions of symbiosis. This implies that regulation of carbon exchange is by the fungal partner, not by the host.

2.3. The role of hexoses in controlling plant–fungus interaction

The supply of glucose by the host appears to affect fungal gene expression. Most probably this is not due to glucose itself but rather to a product of fungal glucose metabolism. This assumption stems from experiments with glucose analogues. Neither 3-*O*-methyl glucose, which is taken up by mycelia but not metabolised, nor 2-desoxyglucose, which is taken up and phosphorylated but then not further metabolised, are able to mimic the effect of glucose [82].

The hexose transporter gene is not the only gene responding to glucose availability. Differential screening of cDNA libraries from sterile cultures of *A. muscaria* and of mycorrhizas formed between *A. muscaria* and *P. abies* delivered a range of clones which were up-regulated (as shown for AmST1), but also down-regulated by a continuous supply of hexoses above the 5 mM threshold. One of these clones obtained from *A. muscaria* could be identified as a key enzyme of secondary metabolism, phenylalanine ammonia lyase (AmPAL; [80]). The transcript of this gene was abundant in hyphae grown at low external glucose concentrations but exhibited a 30-fold decrease in amount in hyphae cultured at glucose concentrations above 2 mM. In contrast to AmST1, this gene is possibly regulated in a hexokinase-dependent manner, although control via later steps in glycolysis can also be assumed; amino acids which can be used as nitrogen sources had a similar effect. As ammonium was effective, altered gene expression possible resulted from amino acid catabolism in the absence of sugar supply.

Phenylalanine ammonia lyase (PAL) is a key enzyme of secondary metabolism and thus of the production of phenolic compounds. In plants, phenolics are widely used in protection against pathogenic invasion. ECM forming

fungi have also been reported to use phenolic compounds for protection of themselves and also of their host plant against bacterial or fungal attacks [38]. We hypothesise that free living mycelia (i.e. low glucose supply) produce more of these protective compounds than those involved in symbiotic structures. We further expect a gradient in expression of the AmPAL gene in mycorrhizae according to a gradient of hexoses from the Hartig net toward the outer hyphal mantle of an ECM. This could cause high levels of phenolic compounds within fungal cells exposed to the soil (hyphal mantle) versus low amounts in the area of the Hartig net, where sugars are abundant and no protection is necessary.

2.4. *The effect of nitrogen supply on plant–fungus interaction*

In many forest ecosystems, rates of nitrogen mineralisation of litter are low. Consequently, supply of inorganic nitrogen is often limiting [95]. In addition, nitrification is usually slow and the poorly mobile ammonium ion predominates as an inorganic nitrogen source [65]. Association with ectomycorrhizal fungi can improve nitrogen acquisition by facilitating access to organic nitrogen sources [98] and by increasing uptake of ammonium [100] via extensive growth of soil mycelia and circumvention of ammonium depletion zones. Therefore, mycorrhiza formation is an important strategy for improving the development and competitive ability of forest trees [9,58].

The ability of ectomycorrhizal fungi to take up inorganic nitrogen and transport nitrogen-containing solutes to their host plant is well established ([33,76,88]; for reviews, see [17,35]). In accordance with the predominant occurrence of ammonium in the soil, most ectomycorrhizal fungi grow better with ammonium than with nitrate as the nitrogen source in pure culture [34,36]. Their ability to assimilate ammonium has been shown in several ways [16,35,73].

Dark fixation of CO_2, which supports amino acid formation via anaplerosis, can be shifted between the partners of symbiosis. In plants, phosphoenolpyruvate carboxylase (PEPC) is an important anaplerotic enzyme. Under regular ammonium supply, mycorrhiza formation causes a significant decrease of both PEPC activity and PEPC protein (Western blot) of roots of spruce seedlings in comparison to the non-mycorrhizal condition [125]. A microhistochemical analysis showed that this effect was most pronounced in the area of most intense interaction of both partners. In parallel, the rates of carboxylation of whole mycorrhizas were significantly increased, implying that under conditions of symbiosis, ammonium assimilation and thus anaplerotic carboxylation is shifted from the root to the fungus [125]. This is presumably the basis for the transfer of amino acids such as glutamine, glutamate or asparagine from the fungus to the host [17].

In recent studies, it was possible to identify a cDNA

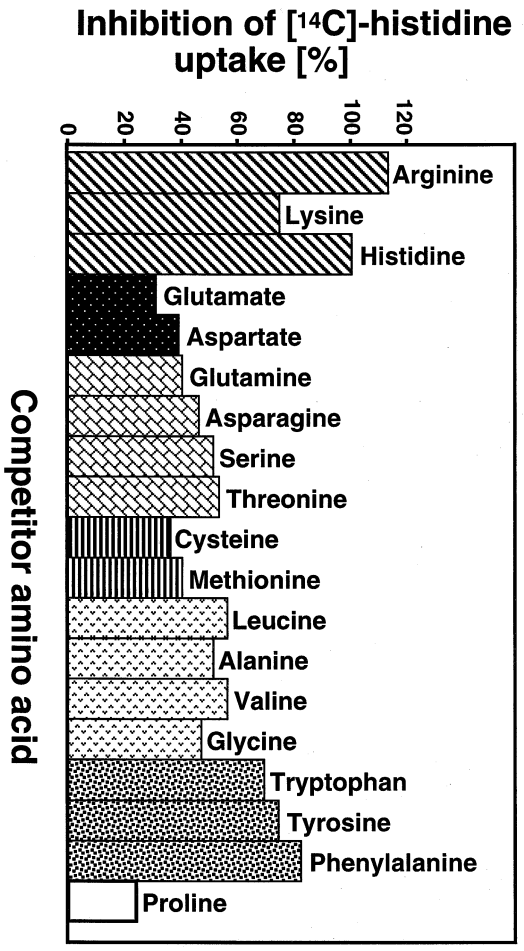

Fig. 2. Uptake efficiency of different amino acids compared to histidine. A yeast strain, expressing *AmAAP1*, was incubated with a mixture of [^{14}C]histidine (25 μM) and an unlabelled amino acid (125 μM). The competition between the uptake of labelled histidine and unlabelled amino acids was measured as % of [^{14}C]histidine uptake. The inhibition of [^{14}C]histidine uptake in the presence of non-labelled histidine was set to 100%.

encoding a fungal amino acid transporter (AmAAP1) from *A. muscaria*/*Populus hybr.* ECM. The expression of this transporter gene was enhanced 10-fold in the absence of a nitrogen source which could be utilised by the fungus. The expression of AmAAP1 in a yeast mutant revealed its function as a high affinity amino acid transporter with a broad substrate spectrum (Fig. 2). AmAAP1 transports a wide range of amino acids with K_M values between 22 μM for histidine and up to 100 μM for proline. The mode of expression suggests that such a transport system is more involved in the uptake of amino acids rather than in the export to the host plant.

A surplus of N availability in the soil can affect fungus/host interaction. In fertiliser experiments, carried out both in natural conifer forests and in pot experiments, a reduction of the mycorrhizal development at elevated levels of N has been reported [6,119,123]. This is most probably a consequence of decreased photoassimilate allocation to the root if photoassimilate production is limited.

It is common knowledge that in plants, a surplus of nitrogen can divert photoassimilates away from the formation of storage or transport carbohydrates such as starch and sucrose, respectively, into amino acid or protein synthesis (for a review, see [19]). Regulatory steps involved are fructose 1,6-bisphosphatase (FBPase) and PEPC (see above). FBPase, a key enzyme of gluconeogenesis, is inhibited by fructose 2,6-biphosphate (see above). Needles of Norway spruce seedlings grown in a semi-hydroponic cultivation system under increased supply of inorganic nitrogen exhibited a 6-fold increase in protein-specific PEPC activity and three times more fructose 2,6-biphosphate than control plants [124]. In parallel, the content of starch was decreased. Root samples exhibited similar responses. This can be taken as evidence that, under limited photosynthetic carbon fixation, an increase of nitrogen supply causes a switch from gluconeogenesis, i.e. starch or sucrose formation, to glycolysis. Decreased rates of sucrose formation will adversely affect delivery of carbon to sink organs, such as mycorrhizal roots. This should have considerable impact on the symbiotic plant–fungus interaction. In order to test this assumption, pot experiments with 3-year-old Norway spruce plants were performed, where N-poor forest soil was supplemented with nitrogen [120]. As in the hydroponic culture system, the anaplerotic key enzyme, PEPC, was significantly increased in both needles and roots while starch was decreased. This implies that under these conditions (in which the potted plants had formed mycorrhizas), ammonium, but not amino acids, was transferred to the plant. In parallel, root-associated fungal biomass, as determined via ergosterol, was only half of that of controls. More importantly, the contents of fungus-specific metabolites such as mannitol and trehalose were even more reduced. This clearly showed that a decreased rate of carbon allocation to the mycorrhiza decreased the number of living hyphae, and caused starvation of the ones still physiologically active. There is, however, also evidence that the fungal partner can store a surplus of assimilated nitrogen in its vacuoles. This is balanced with the fungal glycogen content when rates of photoassimilation by the host plants are increased under elevated CO_2 [116].

2.5. *Regulation of mycorrhiza formation by C/N interaction?*

The studies of gene expression described above clearly show that host carbohydrates have a profound effect on the fungus in that hexose availability at the plant–fungus interface causes changes in levels of fungus-specific transcripts. The genes examined so far are most probably representative of many more, the expression of which is altered in a hexose-dependent manner. Therefore, hexose supply will most certainly play an important role in the development of a functional ECM.

Increased supply of CO_2 for example can increase the source properties of leaves and will thus improve provision of photoassimilates to the mycorrhizal sink, with the consequences of increased mycorrhiza formation and, finally, better water and nutrient exploitation [57,67,68,71,85].

Increased nitrogen supply on the other hand will reduce mycorrhiza formation under limiting carbon supply. As discussed in detail above, under these conditions, photoassimilates will preferentially be directed to host glycolysis and in due course support synthesis of amino acids, rather than sucrose formation and carbon export [116]. We assume that under these conditions, the availability of hexoses in the Hartig net will be rather limited and, by decreasing below the threshold concentration, hexose-regulated expression of fungal genes will be halted. In consequence, the symbiotic interaction should disintegrate and this is clearly shown by the decrease of fungal markers (ergosterol, mannitol, trehalose) in pot experiments with supplemented nitrogen [120]. We thus conclude that a surplus of nitrogen in the soil will have a negative impact on the plant on the longer run; roots with a greatly reduced degree of mycorrhization can suffer from nutrient imbalances as the acquisition of other nutrients by fungal hyphae can become limiting. In addition, plants will probably be much more sensitive to drought stress and to attacks by root pathogens.

3. Molecular approaches of investigation of diversity in ECM in the field

As mentioned in Section 1, 5000–6000 fungal species are involved in ECM. Many of these fungi are not easy to cultivate, so that their patterns of distribution have to be detected [11] directly. This is impossible or at the most very difficult using description of ECM morphotypes in complex habitats [3,90] or inferred from the distribution of fruitbodies, which is a long task due to the non-regular and unpredictable fruitbody production. Nonetheless, analyses of diversity and distribution patterns of ECM are crucial to understand their ecological role as they reflect the heterogeneous availability of nutrient resources in soils and the stabilisation role of the symbiosis for plant communities. PCR-based molecular markers allowing differentiation between species or genotypes have become rapidly indispensable for ecological surveys of the diversity of ECM fungi. An impressive number of PCR-based techniques and markers are now available to detect DNA polymorphism in fungi, each available technique exhibiting differently adapted power to resolve genetic differences. In this part, we will present recent results in analysing the nuclear ribosomal internal transcribed spacers (ITS) region among ECM species. According to the analysis performed after PCR amplification of this region, different levels of differentiation can be reached. Furthermore, the procedures have now been standardised to allow routine analysis of a broad range of ECM taxa in different forms.

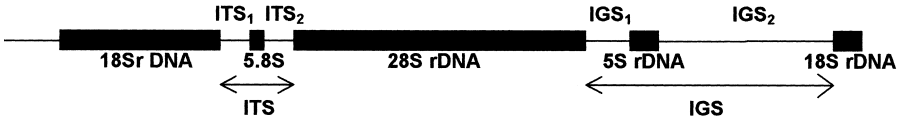

Fig. 3. Schematic representation of the rDNA with the ITS and the IGS. The dark bars represent genes with highly conserved sequences, whilst the sequences of the spacers (thin lines) are more variable.

3.1. Structure and evolution of ribosomal DNA (rDNA)

The genomic region encoding ribosomal RNA is well suited to design nucleic acid markers at different taxonomic levels because it contains genes and spacers (Fig. 3) which evolved at different rates. Thus, interspecies comparisons of the rDNA unit reveal a high degree of conservation in coding regions (18, 5.8, 25 and 5S genes) and considerable sequence differences in the spacers (ITS and intergeneric spacer (IGS)). In all eukaryotes, the ribosomal genes are clustered in tandemly repeated units subject to a continuous process of homogenisation that imparts a greater within- than between-species homogeneity [7]. But in parallel, disparity at intra-specific and intra-individual may occur [21]. For all these reasons, the rDNA offers a set of target regions with differing levels of genetic resolution from groups like families to populations.

3.2. The rDNA ITS as a species marker for ectomycorrhizal fungi

Within the rDNA, the region often referred to as ITS contains the 3′ end of the 18S gene, the ITS1 spacer, the 5.8S gene, the ITS2 spacer and the 5′ end of the 28S gene (Fig. 3).

Traditionally, distinguishing characters for fungal species recognition are mainly phenotypic, with similar individuals designated as a species. Recent years have seen a flood of sequence data in the ITS applied to questions in the systematics, phylogeny and ecology of fungi. Sequence analysis generally reveals a high degree of variation between species, but a low intra-specific variation [31,42,66,69,74]. For example, we found that pair wise divergence between ITS sequences ranged from 1% between two *Tricholoma populinum* isolates to 15% between two species of the same genus (e.g. *T. populinum* and *Tricholoma scalpturatum*). Conflicts between phenotypic and genetic characters have appeared in some cases [32,64,128]. These problems could be anticipated because: (i) some fungal groups exhibit a limited number of characters to determine species boundaries, (ii) intra-specific phenotypic plasticity is often unknown, (iii) convergent evolution seems to be rather frequent, and (iv) cryptic species (reproductively isolated but not separated morphologically) appear to be common in many genera of fungi. Based on the ITS data, a careful re-examination of the morphology was done in several ectomycorrhizal taxa such as *Pisolithus*, *Sarcodon* and *Rhizopogon* [5,59,74,75,105]. These examples have shown that the genetic data are correct and that a re-classification of phenotypic species should be proposed for these fungi. Alternatively, the ITS may fail to separate well recognised phenotypic species. For example, a lack of phylogenetic resolution has been observed in the genus *Hebeloma* based on sequences in the ITS region [2]. This low level of genetic divergence among species may indicate a recent radiation event with sibling taxa that have not been separated sufficiently to diverge in the ITS region. Another potential complication comes from the presence of intra-specific and intra-individual variation which may sometimes affect the ability to identify species using simple molecular methods such as restriction fragment length polymorphism analysis of PCR products (ITS-RFLPs). For example, two ITS-RFLP variants have been detected in dikaryotic isolates of *Hebeloma mesophaeum* [1]. These two types were located on homologous chromosomes in the different nuclei of the dikaryons.

In spite of these problems, the ITS has considerable utility to identify distantly related species or groups of related ectomycorrhizal fungi within a species, and ITS sequence data are accumulating very fast. When intra-specific and intra-individual differences are a complication, the identification of sequence and secondary structure patterns across groups of organisms may help to predict which sequence changes best highlight species- or group-specific differences. Future research may include other target sequences such as the V4, V6 and V9 domains of the mitochondrial small-subunit rRNA gene which also hold potential for diagnostic markers at the species level. For example, comparative studies of these domains carried out in two saprophytic genera, *Agrocybe* and *Pleurotus*, have shown that intra-specific variation was minimal while important sequence differences were detected between species [44,45].

3.3. Experimental procedure

Any technique for routine analysis of heterogeneous field material absolutely requires a description of the best available version of standard experimental procedures.

3.3.1. Source material

3.3.1.1. Fruitbodies. DNA is routinely extracted from specimens cut in small pieces and air-dried overnight at 40–50°C. For long term DNA preservation, lyophilisation of fruitbodies is preferable.

3.3.1.2. ECM. Fresh root samples can be processed within a few days or preserved in solutions containing detergents or alcohol. No reduction in amplification yields is noted when DNA is extracted from mycorrhizas preserved in 30% ethanol at 4°C for 1 month or even at least for 3 years in 2× hexacetyltrimethylammonium bromide (CTAB) extraction buffer at 20°C [39]. Pure or diluted acetone also allows long conservation [37].

3.3.2. DNA preparation and PCR amplification

The amount of DNA template necessary for PCR amplification is so small that, in theory, any simple extraction method should work. Grinding of single mycorrhizal tip, small pieces of hymenium or mycelium in microfuge tubes is combined with freeze-thawing and CTAB extraction to break the cell wall and allows the DNA to go into solution ([39]; J.Y. Charcosset and M. Gardes, unpublished results). Polysaccharides, tannins and proteins are extracted with chloroform, and an overnight isopropanol precipitation allows recovery of the greatest amount of DNA. Amplification conditions are as described by Gardes and Bruns [40]. The sensitivity of detection is high (down to 2 pg total DNA in 25 μl reaction) because the ITS is a multi-copy target.

3.4. Ecological applications

3.4.1. Detection of selected species for autecological studies

PCR involves hybridisation of oligonucleotide primers that flank the DNA to be amplified. Length, G+C content, number, position and quality of mismatches are taken into account for the design of taxon-specific primers [12,39]. If sufficient sequence variation exists in the ITS, it is possible to select species-specific primers. Amicucci et al. [4], for example, found adequate differences in the ITS region of five closely related species of white truffles to design pairs of primers that specifically amplify the DNA of each species. A rapid identification of the fungal symbiont was carried out in a single-step PCR amplification from DNAs of fruitbody, mycelium and ECM. Eberhart et al. [29] used the same approach to differentiate two species of *Tylospora* in spruce ECM. Several species can be detected at the same time in multiplex PCR using a mixture of species-specific primers [78,87]. This molecular method is easy to perform and does not require sophisticated equipment. Potential difficulties and ways to avoid problems in multiplex PCR have been described in detail by Henegariu et al. [52]. We are currently screening several hundred poplar mycorrhizas by multiplex PCR [18], to assess the persistence and dissemination of two *Tricholoma* species under field conditions (M. Gardes and J.Y. Charcosset, unpublished results). A species-diagnostic assay was developed for *T. scalpturatum* and *T. populinum*. Clusters of 2–5 non-homologous nucleotides were identified in their ITS. These differences permitted the synthesis of oligonucleotides that hybridise differentially and produced amplified fragments of diagnostic size for each species.

Less specific fungal primers (i.e. ITS1-F, ITS4-B, NL6Amun, NL6Bmun) that hybridise to ribosomal sequences conserved across a broad range of fungal taxa are also available [30,39]. Species are differentiated in a second step by various methods such as restriction enzyme analysis (see Section 3.3.2) or species-specific oligonucleotide probing in a dot-blot Southern hybridisation assay. We are routinely using primer ITS1-F along with either primer ITS4 or primer ITS4-B to amplify the ITS region from mycorrhizas and fruitbodies. Both primer pairs discriminate against plant DNA [39]. Neither amplification nor discrimination problems have been encountered with these oligonucleotides. However, faint amplification products have been obtained with ITS4-B, a primer intended to be specific for basidiomycetes, with some 'lower' taxa such as *Rhizoctonia* associated to orchids [113].

3.4.2. Detection of ITS variation in the study of communities

A community is a system of interplaying species populations which shows differentiation in space and time. Patterns of species distribution develop in response to differences in environment and interactions among species. Communities also differ in species richness; depauperate habitats often have harsh and/or unpredictable abiotic conditions. In the last 5 years, the use of molecular markers in the ribosomal genes and spacers has contributed to significant progress in determining the species which are present on roots and how they position themselves within the community. Considerable variation is observed among species in the relationships between fruitbodies and mycorrhizas [25,41,60,92]. Some mycorrhiza producers fruit rarely, others are frequently encountered on the basis of fruitbody surveys. Fruitbodies are poor indicators of the below ground community because they are produced very irregularly, ephemeral and difficult to detect and thus reflect very partially the presence of ECM [117]. However, mycorrhiza surveys must also be interpreted with caution because the extent of ECM turnover is not well known and species may exhibit substantial spatial variation in mycorrhiza production [114]. This emphasises the need of sampling strategies that adequately reflect the heterogeneity of distribution patterns.

An easily used and cost-effective technique to detect polymorphism consists in digesting the amplified products with restriction enzymes followed by gel electrophoresis. This method requires minimal equipment, relatively little molecular expertise, and can rapidly establish species-specific restriction patterns (ITS-RFLPs). A first step consists in randomly selecting four-base restriction enzymes and to assay variation on a subset of samples. DNAs from identified fruitbodies collected on the study site are used to select two or three enzymes that detect variable restriction. These are then used to screen variation in all samples and

the data are combined to generate groups of fungi with identical RFLP patterns for all the enzymes (= ITS-RFLP types). Due to the high degree of interspecific sequence variation in the ITS, a low number of enzymes appears sufficient to separate distantly related species and generally species within the same genus [92]. Several authors also observed that fungi with identical RFLP patterns usually have identical or related DNA sequences ([64,83]; J.Y. Charcosset et al., unpublished results). For example, about 6% of the sequence differed in the ITS region of two *Cortinarius* species that were indistinguishable with three different restriction enzymes [64]. In cases where no match is observed between ITS-RFLP patterns from mycorrhizas and from fruitbodies, phylogenetic placement of the unmatched samples may be inferred from sequence databases. Species identification based on the ITS is still limited because only a small number of ectomycorrhizal species (less than 100) have been sequenced. More complete databases which provide family to generic placements have been assembled in both the mitochondrial large subunit ribosomal gene and the nuclear 28S ribosomal gene [13,114]. Identification at the family or genus level with this method helps to narrow down the search for successful ITS-RFLP matches. Sufficient sequence variation also exists in the 5.8S rRNA gene to allow differentiation of broad groups of organisms. Cullings and Vogler [23] used this database to identify a discomycete that forms abundant ECM in burned stands of *Pinus contorta*.

3.4.3. Some perspectives

Mycorrhizal ecologists are interested in recognising fungal symbionts at all stages of development including fruitbodies, mycorrhizas and mycelium. The application of molecular markers is already beginning to transform our understanding of the factors that influence the evolution of mycorrhizal symbiosis at different ecological and evolutionary scales.

3.4.3.1. Host specialisation. Selection by the host may be an important force shaping community structure at a local scale but extensive data gathering in natural communities is only beginning to emerge [24,54,55,113]. Among ECM formers, a large spectrum of host specialisation is found. Some genera and species associate with roots of a broad range of deciduous and coniferous trees and shrubs, whilst others are apparently restricted to association with a few plant species [79]. Complex patterns of specialisation are observed because interaction diversity is encountered at different levels. For example, *Hebeloma*, a widely distributed genus, is commonly thought to be a generalist as opposed to the genus *Suillus* which is almost exclusively associated with *Pinaceae*. However, phylogenetic analysis in the ITS of *Hebeloma* showed that this genus is clearly composed of two major groups of species. One exhibits a clear preference to *Salicaceae*, while the other has a broad host range but is rarely found with *Salicaceae* [2]. Similarly, widely distributed species thought to be generalists are probably complexes of specialised populations. Restriction of host range may occur if two possible partners are geographically separated, if they do not meet (as a result of different phenologies, for example), or if they are incompatible metabolically and genetically. As ecological conditions change, some interactions may evolve rapidly towards decreased or increased specialisation. Fungi receive most of their energy from the host plant. Environments which experience a high fluctuation of host populations will perhaps select against fungal taxa that colonise inconsistent or unpredictable hosts. Exploration of this domain only becomes possible by using the molecular biological tools described above.

3.4.3.2. Impact of disturbances. Increased attention is also given to the spatial variability of fungal communities in field conditions and how these communities evolve as ecological conditions change through disturbances. Recent studies point to the impact of fire, insect herbivory and fertilisation on communities of ectomycorrhizal fungi [8,43,60,61,63,114]. These observational studies have highlighted various aspects of interactions between fungal species and the importance of plant–fungal feedbacks. However, many more comparative studies are required before generalisations can be made on the processes by which mycorrhiza formers establish and persist under varying environmental conditions. After the detection of patterns, there is still much work to be done to establish specific causes for the observed distribution patterns and to elucidate the functional roles of these fungi. Permanent plots should be established to monitor the responses of individual fungal species after habitat modification and in relation to plant stand development. There is a multitude of ectomycorrhizal fungal species in a given ecosystem but it should be possible to re-classify the species involved into a small number of groups relevant to their biological and ecological traits, ranging from dispersal and foraging abilities to stress tolerance. These epidemiological criteria determine differences in competitive abilities of ECM fungi and form the basis of any functional classification [84]. Other attributes of functional significance are related to the effectiveness of nutrient capture and mobilisation. Such classifications need to be developed and are necessary if ecologists are to generate predictions about the conservation of biodiversity or the elasticity of ecosystems against modifications.

3.4.3.3. Relation between structural and functional diversity. According to Read [95], it is to be expected that the population structure of the ECM fungi reflects a close adaptation to the available N resources at a specific location. Attempts to investigate this kind of relationship were performed in a relatively simple forest ecosystem comprising an alder stand. The first step was to analyse ECM diversity by both morphological and molecular methods

[91,92]. The second step, the analysis of functional diversity, necessitated isolation of ECM fungi, which appeared to be possible only for two ECM species from 15 present at the stands [89,90]. By chance, these two forms represented a high percentage of the ECM at the locations. The genetic relationship between the isolates was assessed by ITS sequencing (K. Pritsch et al. unpublished data). The potential of the isolates to metabolise diverse sources of organic and inorganic N was assessed in pure culture experiments. At the inter-specific level, the pattern of N-source utilisation corresponded to the genetic distance found in the ITS analysis. Such a relationship could not be shown clearly at the intra-specific level, essentially because of the low growth rate of the isolates in pure culture that did not reveal small quantitative variations. This kind of analysis illustrates the difficulty in relating a pattern of ECM distribution in the field to the capability of populations to mobilise different sources of nitrogen. It also underlines the difficulty in drawing conclusions for field conditions on the basis of tests in pure culture.

4. Concluding remarks

4.1. The advances

Symbiotic associations have long been neglected in ecology. Until recently, certain ecologists even considered that symbioses cannot have any ecological importance, because they are formed by partners with biological features which differ too much (for example, propagation rates) and should therefore be basically unable to adapt to fluctuating conditions in ecosystems ([10]; see also discussion in [28]). This consideration by the Newtonian ecological school is clearly refuted by the effective role that symbioses like ECM obviously play in nature [62,96].

In Section 2, we presented recent advances toward the understanding of mechanisms involved in formation and regulation of ectomycorrhizal symbioses. This part of the review focused on the C-translocation from the plant to the fungal partner and on its regulation by nitrogen supply. The results presented are based on experimentation with model systems under laboratory conditions. They contribute to our understanding of functional aspects as well as of the capability of ECM to adapt to changing conditions in the ecosystem. At the moment, we just get a glimpse at the complex systems of plant/fungus interaction in which the supply of hexoses appears to have a key function. We do not yet know whether hexoses or products of metabolic conversion by the fungus are the signal, and which *cis*/*trans* elements are involved in the regulation of partner-specific gene expression. Furthermore, it is still difficult to transfer data obtained with sterile laboratory systems to symbioses under field conditions. Here, many more organisms are involved in making nutrients available for both symbiotic fungi and their host plants. In this context, most important and largely neglected role is that of soil bacteria. Recent advances in the Tübingen laboratory clearly indicate a significant contribution of these bacteria to the development of ECM fungi (Maier et al., unpublished data).

The rapid development of molecular tools in the investigation of ECM population structure, reported in Section 3, constitutes a promising way to move from an empirical description to a causal explanation of ECM function in the field. This can be achieved by correlating analyses of ECM population structure to soil or microclimatic factors. In this regard, the question of the sampling intensity which has briefly been mentioned above is crucial. The resolution level of molecular methods enables the rapid but precise characterisation of sufficient mycorrhizal rootlets to envisage population analysis in ECM based on geostatistical methods. This could dramatically enhance the significance of relationships established between the distribution of ECM population in a given ecosystem and the variation of ecological factors, and constitutes a challenge for mycorrhizal field research in the years to come.

Molecular tools can also help to investigate the impact of the symbiosis in the biology of ECM fungi and for their evolution per se. The importance of dikaryons for certain ECM basidiomycetes in order to form symbioses has been described [115]. Somatic interactions have long been recognised to be crucial for colonisation of complex substrates like wood or soil by saprophytic fungi [94]. They may determine the place of mycorrhizal symbiosis in the life strategy of fungi [14]. Analyses of rDNA (sequencing and RFLP) also allow assessment of the specificity of putative species to certain host plants, to resolve open questions on systematics and analysis of the importance of symbiosis in the evolution of a fungal group [126–128].

4.2. The remaining problems

Integration of both laboratory and field approaches, as described in Sections 2 and 3, principally can highlight ECM functioning in ecosystems. However, difficulties with this integration should be emphasised. For example, the progress described in elucidating regulatory aspects on adapted models under controlled conditions is far from explaining ECM functioning in complex ecosystems. One illustration is given by the question of the photoassimilate allocation. Comparisons performed on laboratory model systems suggest that the time at which the sugar exchange is established during ECM formation could vary according to the fungal species [53]. In ecosystems, trees with differing photosynthetic efficiency are connected via ECM fungi and can exchange carbon. This has been assessed experimentally [97], but it will be difficult to monitor the regulation of this balance under field conditions.

The development of molecular probes allowing the expression of genes involved in metabolic activities to be followed in situ can help to monitor the functions of mi-

cro-organisms in the soil. Such tools are now used with soil bacteria [51] but they will be difficult to optimise in the case of fungi due, for example, to the size and form variation of these organisms. For this reason, direct assessment methods will at the most allow exemplary monitoring of physiological regulation of ECM in soils. Even if not totally satisfying, function analysis of ECM will continue to progress through verification of hypotheses formulated on the basis of investigations under controlled conditions by relating mapping of variations of ecological factors in the field to the distribution of ECM.

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