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Chapter

Conservation of Edible Ectomycorrhizal Mushrooms: Understanding of the ECM Fungi Mediated Carbon and Nitrogen Movement within Forest Ecosystems

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Abstract

Most edible ectomycorrhizal (ECM) mushrooms are currently harvested from nature and many of them are high-priced. Demand for the wild mushrooms as a culinary delicacy has stimulated research that aims to understand (1) the puzzled role that the ECM fungi play in the forest ecosystem, and (2) nutritional and other requirements for fruiting, which is highly variable. In this review, we focus on understanding of the ECM fungi mediated carbon and nitrogen movement between the symbiotic partners and on the interactions with other fungi in forest ecosystems. Thereby, we better understand the diverse nitrogen requirements for edible ECM fungal growth and mushroom fruiting. We attempt to provide a theoretical basis for the future research of edible ECM mushrooms in wild and controlled conditions.

Keywords: culture, cultivation, ectomycorrhizal fungi, edible mushrooms, nitrogen uptake

1. Introduction

Forests play a crucial role in the global environment and economy. Forest-based wood products as well as non-wood forest products have offered remarkable resources and benefits for the well-being of people [1, 2]. A healthy and well-growing forest system is largely dependent on available soil nutrients and efficient nutrient cycling [3, 4], especially nitrogen (N). As we know, nitrogen is a limiting resource for plant growth in many temperate forests.

Nitrogen is necessary for plants. Most crops require N relatively high amounts, but only a small amount of available N is present in soil at a time. A large source of soil N is the atmospheric dinitrogen (N2), the major gas of air (79%) [5]. Only certain microorganisms can bind molecular nitrogen from air. All other organisms need to take up nitrogen from soil. Soil organic matter (especially humus) acts as a storage and supplier of nitrogen for plant roots and microorganisms;

almost 90–95% of soil total nitrogen originates from soil organic matter [6, 7]. Plants acquire N mostly from the inorganic forms such as ammonium and nitrate. However, plants that associate with mycorrhizal fungi are considered to have greater access to organic nitrogen pools when compared to non-mycorrhizal plants [5].

ECM fungi play an important role in the nutrient cycle of terrestrial ecosystems. Especially in a forest poor in nutrients, the growth of trees depends on the existence of mycorrhizal fungi. The value of ECM fungi is evaluated from the global framework. ECM fungi provide hidden biological fertilizers for increasing plant biomass, conventional afforestation, and ecosystem restoration practices; they also control soil pathogens [8–10].

In addition to benefits for forests, many ECM fungi produce edible mushrooms that are widely appreciated for their nutritional, medicinal, and gastronomic properties [11]. One of the major challenges of the twenty-first century is to produce sufficient food. From that perspective, wild mushrooms as non-wood forest products are getting more and more attention globally [12]. It would be convenient if these mushrooms could be cultivated. However, most edible ECM mushrooms can only be collected from nature and not cultivated artificially [11]. The main obstacle to the cultivation of edible ECM mushroom is their need to be associated with a host plant in plantations. The association is obligatory for the successful growth and fruiting of the mushrooms. The unanimous discussion of the nutritional growth requirements of ECM edible fungi is a topic of interest for scientists.

An in-depth understanding of the nutritional requirements of ECM fungi and the role of ECM fungi in nutrient cycling, particularly in ECM fungi mediating carbon and nitrogen movement within forest ecosystems will be summarized in this chapter. The nutritional requirements to successfully culture and cultivate ECM fungi will be discussed.

2. Ectomycorrhizal fungi

2.1 Ectomycorrhizal fungi

Ectomycorrhizal fungi are found in association with the roots of most forest trees throughout the world. ECM fungi form obligate symbioses with many of the dominant trees in temperate and boreal forest, as well as in some tropical forests. ECM fungi do not penetrate their host's cell walls. Instead, they form an entirely intercellular interface, known as Hartig net, consisting of highly branched hyphae that forms a latticework between epidermal and cortical cells [13]. Hartig net provides a large surface area for the two symbiotic partners and it is the site of nutrient exchange. Carbon (C) is transported to the fungus from a tree that receives limiting nutrients in exchange. The fungus can transport nutrients beyond the nutrient depletion zone surrounding the host's root system and release from immobilized sources inaccessible to the plant [13, 14]. ECM fungi are thus regarded as key elements of forest nutrient cycles and as strong drivers of forest ecosystem processes [15].

Most (86%) terrestrial plant species obtain mineral nutrients through mycorrhizal symbionts as estimated using taxonomic and ecological extrapolation methods [16]. An estimate of ECM fungal species richness is likely between 20,000 and 25,000 [16, 17]. These ECM fungi belong to more than 80 independently evolved lineages and to more than 250 genera, mainly in Basidiomycetes and Ascomycetes [18].

2.2 General roles of ectomycorrhizal fungi in forest ecosystems

Ectomycorrhizal fungi are essential contributors in forest ecosystems by forming beneficial symbiosis plants. These fungi drive forest soil processes such as soil organic matter decomposition, nutrient cycling, and carbon sequestration [19–21].

ECM fungi have the ability to provide hosts not only nitrogen but a variety of other major nutrients, including phosphorus, potassium, calcium, magnesium, sulfur, as well as micronutrients such as iron, zinc, copper, and manganese. However, they are often ignored because N is the main growth-limiting element in many forest ecosystems, particularly in the Northern Hemisphere [19, 22, 23]. In addition to nutrients, trees receive several other benefits. First, the resistance of trees against pathogens is improved due to the mycelial network [24]. Second, the ECM mycelial networks are involved in water transport [25]. Third, ECM fungi can relieve salt and heavy metal stress of the host plants [9]. The benefits that the ECM fungi offer are complicatedly regulated by the host type, ECM species, as well as climatic and environmental conditions. Recently, a study based on a climate change model predicted that the global abundance of ECM-associated trees will decline by 10% by the end of 2070, and the majority of this will take place in boreal and temperate ecotones [26]. Therefore, the conservation of ECM fungi should be taken as an important issue.

2.3 Structure of ectomycorrhizas is diverse

Fungal mycelium has been estimated as one of the largest living organisms on Earth [27]. Hyphae is composed of fungal mycelium and other structures including rhizomorphs. Rhizomorphs are structures through which fungi can spread in their environment and search for new substrates to colonize. The structure of ectomycorrhiza is diverse. Agerer [28] proposed that ECM mycelia systems influence on their patterns of differentiation and putative ecological importance. Mycorrhizal fungi have been classified into four exploitation types depending on the extent of hyphal development: contact, short-distance, medium-distance, and long-distance.

ECM fungi are characterized according to the water repellence of the mycelium. Fungi vary from extremely hydrophobic to extremely hydrophilic types [29]. All fungal growth parameters such as hyphal hydrophilicity, presence of rhizomorphs, and mat formation correspond together to how fungi interact with and exploit the environment [28, 30]. The function of extraradical mycelia of ECM fungi is the transportation of nutrients between plant and soil environment [13, 31].

Ectomycorrhizas differ in their ability to take up and transport nutrients, and thus, promote tree growth [32, 33]. The differences in ECM effectiveness are often species specific or even strain specific [34]. It is evident that the amount and differentiation of extraradical mycelium is an important ecological factor for tree performance [35–37] and soil nutrition [38].

3. Contribution of ectomycorrhizal fungi to nitrogen cycling in forest ecosystems

3.1 Forms of nitrogen in forest soil

The major N sources in the forest floor can be divided into external and internal sources. Atmospheric nitrogen deposition is an external source, and the living organisms and their decomposition products are an internal source [39, 40].

Ammonium and nitrate are the two major pools of inorganic N. Ammonium is most often the dominant inorganic N pool available to trees in coniferous ecosystems. Nitrate concentrations are usually relatively low in mature forests [41].

Most of the nitrogen in forest soils is bound to organic compounds [42]. It is well known that over 90% of N occurs in organic forms in most surface soils [7, 43]. The forms of organic N can be roughly divided into two categories. (I) Organic residues consisting of undecomposed animal and plant residues and partial decomposition products, and (ii) soil organic matter or humus. The humus is composed of non-humic, easily identifiable compounds (e.g. amino acids, carbohydrates, nucleic acids, etc.) and complex humic substances, such as high-molecular-weight amorphous and aromatic compounds, formed during the decomposition process. The importance of humus is widely recognized in maintaining and improving soil fertility [7].

The distribution of major N compounds was investigated in different climatic and geological conditions including arctic, cool, temperate, subtropical, and tropical climates early [44]. The results indicated that about 33–42% of soil N occurs as free and protein amino acids. The amino acid composition of all soils, however, was remarkably similar. The composition and concentration of amino acids has shown generally constant throughout the growing season [45], which suggests that amino acids originate from a common source or through similar biochemical processes. However, the distribution of N compounds at different regions seems to be related to decomposition process and as well as forest types [46]. Soil proteins are often not free, they are bound to humic compounds and are not soluble. These N forms cannot directly be used by plants, they need to be depolymerized by microorganisms and converted into plant available monomeric organic or mineral N forms.

3.2 Diversity in nitrogen uptake in Ectomycorrhizal Fungi

Ectomycorrhizas occur widely in forest ecosystems. Most of the terrestrial plant species are in symbiosis with mycorrhizal fungi, about 3% of them are ectomycorrhizal. The most common tree species belong to Pinaceae, Salicaceae, Betulaceae, Fagaceae and Myrtaceae [13, 47]. The general mechanism of ECM fungi to improve plant nutrition is the so called Hartig net structure that increase the surface area of roots to absorb nutrients.

Ectomycorrhizal fungi are able to take up both inorganic and organic forms of N. Ammonium is generally recognized as the most readily utilizable form for most ECM fungi when studied in mycelial cultures [48, 49] or with ECM roots in vitro and in the field [50]. Nygren and colleagues [51] demonstrated that 68 species of ECM fungi used nitrate as the sole N source in a pure culture. However, the pure culture conditions do not reflect the N preference of ECM fungi in nature [52]. *Laccaria laccata* was shown to uptake nitrate and transfer it to the host plant when in nitrate-rich conditions [53].

In other studies, ECM seedlings demonstrate a strong preference for amino acids over ammonium [54]. Already in 1953, Melin and Nilsson [55] demonstrated that ^{15}N labelled glutamate was absorbed by the mycelium of *Boletus variegatus*, and that the nitrogen was transferred to the shoots of pine seedlings that had been infected with the fungi in an aseptic culture. Many ECM fungi are able to grow with amino acids as the N source in pure culture and also in association with host trees [56–59].

The capacities of ECM fungi to mineralize organic N differ. Abuzinadah and Read [60] found that ECM fungi such as *Suillus bovinus*, *Amanita muscaria*, *Paxillus involutus*, *Cenococcum geophilum*, and *Rhizopogon roseolus* were able to use peptides and proteins as their sole N sources. In contrast, *Laccaria laccata* and *Lactarius rufus* had little ability to grow with peptides and proteins but they grew well with

ammonium. It was further demonstrated that different fungal species, even different strains had different abilities to utilize organic N and/or transfer the assimilated N to their host plants [60]. Some ECM fungi might take up the nitrogen compound completely and some break the molecules into smaller organic or inorganic forms. The difference in the ability of ECM fungi to transfer N from chitin, protein, and other organic substances in litter and humus was explained by differences in their enzyme secretion profiles [61].

ECM fungi have several functionally distinct metabolic pathways to transfer N. ECM fungal hyphal morphology, species niche (original living conditions), genetic characteristics and carbon costs to host plants may influence on their capacity to utilize and mineralize organic N.

3.2.1 Mycelium structure determines the efficiency of ECM transport nitrogen

ECM fungal hyphae morphology is diverse. Morphology seems to have a great influence on the hyphal enzymatic ability of ECM fungi. ECM species with hydrophilic ectomycorrhizal hyphae have proteolytic activities and they are adapted to N-limited conditions [62]. In contrast, other ECM fungi with hydrophobic ectomycorrhizal hyphae, similar to many saprotrophic fungi, form aggregated hyphae (rhizomorphs) for long-distance transport of elements. This is presumably an adaptation for patchily distributed resources [63].

In addition to hydrophobicity, another aspect is to consider the size of mycelia. The species that form extensive extraradical mycelia (e.g. *Cortinarius*, *Suillus*, *Tricholoma* species) have different capacity to utilize organic N than those species that form diffuse, spatially limited extraradical mycelia (e.g. *Amanita*, *Lactarius* species). These differences in mycelia are thought to be associated with different reproductive and colonization strategies [58, 62]. It is believed that extensive mycelia are established infrequently, but it is long- living. In contrary, the diffuse mycelia become more stable, usually by spores for the generation, but the mycelia do not persist. The long-living extraradical mycelia is believed to be more efficient to process N than short-living mycelia.

Studies based on the stable N isotope ratios in ECM fungal fruitbodies have provided new insights and evidence for the N sources of ECM fungi. As we know, the relative abundance of stable isotopes in food webs follows from discrimination against heavier isotopes in several biochemical processes [64]. The ratio is useful particularly in studying nitrogen cycling mediated by mycorrhizal fungi [65]. Stable N isotope ratios in ECM fungal fruitbodies showed that those having long-living mycelia exhibited higher $\delta^{15}N$ than those having short-living mycelia [58, 66, 67].

Thus, the signature of ^{15}N in ECM fruitbodies was determined by the morphological characteristics of the mycelia. Another observation revealed by the isotope studies is that ECM fungal species that can utilize organic N exhibited higher $\delta^{15}N$ in their fruitbodies than those that are restricted to mineral N sources [67, 68].

3.2.2 Nitrogen utilization of ECM fungi is related to the nitrogen status of the habitat

The form of nitrogen in the environment influences N mobilization by ECM fungi. The species common in low inorganic N soils grew well with protein, glutamine, and serine whereas species in high inorganic N soils grew well with glutamine, but poorly with protein and serine [67]. Differences among ECM fungal species in their ability to access and take up different N forms indicate that the form and abundance of N in the environment may be a defining factor for ECM fungal species niche [69]. ECM species are selected by the N form that is predominant in

their environment. Recently, an increasing number of studies showed that inorganic N enrichment in forest soils caused by pollution, fertilization or natural causes are leading to a reduction in the level of plant root colonization by ECM fungi, also shift fungal community in soils away from ECM fungi specialized in organic N acquisition to more generalist nitrophilic species and saprotrophs [70–72].

Other studies have concluded that differences in proteolytic activity between the species of ECM fungi could be explained by soil-derived selection pressures. For example, *Hebeloma crustuliniforme* expressed proteolytic activity in the presence of a readily available N source such as ammonium [73]. Ammonium has also been shown to repress the expression of amino-acid transporters and enzymes in N assimilation pathways in ECM fungi [74, 75]. The presence of inorganic N tightly down regulated soil organic matter degradation by *Paxillus involutus* as proved [76]. Such facts suggest that ECM fungal degradation activity would be controlled by environmental factors.

Different ECM species occupy different successional stages in forest development. This seems to be related to the proteolytic activity of fungi. When resource quality declines and organic matter accumulation declines during forest development, fungi with limited proteolytic activity is favored. For the cultivation of edible mushrooms, this means that we should pay attention to the natural preferences of the species for nitrogen uptake. This may concern especially the ECM species that are difficult to cultivate artificially.

3.2.3 Fungal genetic characteristics determines the efficiency of N transition

Recently, advances in genetics and molecular biological techniques have provided better understanding about nitrogen metabolism. The acquisition of inorganic N and the mineralization of organic N by ECM fungi have been proved by many molecular investigations. Ectomycorrhizal fungi encode a number of transporters to acquire nitrate and ammonium from soil, as well as a suite of enzymes and transporters necessary for utilizing organic N sources [77–79]. Ammonium importers such as AMT1, AMT2 and AMT3 have been functionally characterized in several ECM fungal species, such as, *Hebeloma cylindrosporum* [75, 80], *Tuber borchii* [81] and *Amanita muscaria* [82]. Nitrate transporters, such as LbNRT2 in *Laccaria bicolor* [83] and HcNRT2 in *H. cylindrosporum* [84], are also present in ECM genomes allowing N transport.

Ectomycorrhizal fungi have all evolved from their saprotrophic ancestors, and hence, ECM have the ability to decompose organic matter [85, 86]. The utilization of proteins by fungi requires the enzymatic degradation of proteins to peptides and amino acids before cellular uptake. Lindahl and Taylor [87] studied the genetic potential of ECM fungi to produce N-acetylhexosaminidases that hydrolyze chitin to N-acetylglucosamine. Thus, N-acetylglucosamine and amino acids replace ammonium and nitrate as the N sources [19].

Recently, the genomes of ECM fungi were found to contain the same or smaller number of copies of genes coding for secreted N and P targeting hydrolases than saprotrophs, pathogens, or ericoid mycorrhizal fungi [88]. This observation is surprising because the well-documented ability of ECM fungi to hydrolyze organic phosphate compounds and scavenge nitrogen through the degradation of proteins accumulated in litter. Miyauchi and colleagues [88] also showed that the ECM fungus *Paxillus involutus* was able, while assimilating organic N, to significantly modify organic matter with a free-radical-based mechanism similar to that of saprophytic brown-rot fungi [76]. Unlike the saprophytic fungi, *P. involutus* did not show any expression of genes encoding extracellular enzymes needed to metabolize the released C. This suggests that the degradation mechanism of this ECM fungus has evolved to assimilate organic N rather than C.

3.2.4 ECM utilizing organic N in relation to receiving C from trees

ECM fungi are able to breakdown soil organic N with differing efficiencies. It has been found that the uptake of amino acids by mycorrhizal fungi is related to the N content and carbon structure of the amino acid [89]. One hypothesis was proposed that the rate at which mycorrhizal fungi degrade large organic N polymers in soils is also controlled by the plant C resources available to the fungi to construct extracellular enzymes, as well as the bond strength and structural diversity of the target organic N compound although the direct tests of the hypothetical mechanism is still needed. Another study by Näsholm et al. [90] tested a model for C–N exchange between trees and mycorrhizal fungi. They found that ECM fungi transport smaller amounts of absorbed N to trees in N-limited than in N-rich conditions. The study found further that the greater allocation of C from trees to ECM fungi increases N retention into soil mycelium. The growth of these fungi is stimulated, and thus, N is immobilized and sequestered in soil. This mechanism was suggested to drive boreal forests towards a more severe N limitation at low N supply.

ECM fungi have diverse evolutionary origins and they use diverse decomposition mechanisms to access organic nitrogen entrapped in soil organic matter [91]. The timing and magnitude of decomposition activity seem to be controlled by the below-ground nitrogen quality and the above-ground carbon supply. Some ECM fungi might act as decomposers, not primarily to obtain C to their metabolism, but to search for organic N in the absence of readily available inorganic N [76, 92–94].

4. Challenges in establishing edible ectomycorrhizal fungal culture with fruitbody formation

More than thousand species of ECM fungi produce edible mushrooms [95]. Some of them, such as *Amanita caesarea* (Scop.) Pers. *Boletus edulis* Bull., *Cantharellus cibarius* Fr. and *Tricholoma matsutake* (S. Ito and S. Imai) Singer, have economical value on international markets. The problem is that edible ECM fungi are usually more difficult to cultivate than saprophytic fungi because of the symbiotic relationship with a host tree is needed. In the past few decades, significant progress has been made in the cultivation of some fungi, such as *Lactarius deliciosus* (L.) Gray [96–98], *Lactarius hatsudake* Nobuj. Tanaka [99], *Suillus granulatus* (L.) Roussel [96], *Rhizopogon roseolus* (Corda) Th. Fr. [100], and *Lyophyllum shimeji* (Kawam.) Hongo [101]. In controlled conditions, however, the successful fruitbody or primordium formations are limited. Most of edible ECM fungi still cannot be cultivated. The major issues that need to be understood are the trophic relationships, biotic, edaphic, and climatic requirements for each mushroom. In this review, we focus on the nitrogen acquisition of edible ECM fungi for their mycelial culture and its effect on fruitbody formation. Secondly, we take *T. matsutake* as an example and discuss in detail about its ability to acquire nitrogen, its preferences, and possible strategies. Finally, we discuss about the further challenges – to conserve proper ecological conditions for edible ECM fungi to grow.

4.1 Nitrogen sources in edible ECM fungal cultures

We summarize the nitrogen sources used in mycelium culture and the cultivation experiments of edible ECM fungi in combination with ECM fungal morphological characteristics reported from the published studies (**Table 1**). As known, most edible ECM fungi are difficult for cultivation so far. We could get some hints for the ECM cultivation from experimentally observed nitrogen preferences

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
Amanita					Hi	Medium-smooth	3.1 ± 0.5 (35)	[102]
A. caesarea	NH_4^+ (poor on orgN)			[103]				
Boletus					Ho	Long	5.8 ± 1.0 (17)	[102]
							8.66 (1)	[104]
B. edulis		orgN		[105]				
B. reticulatus			NH_4^+ and orgN	[106]				
Boletus sp.			NH_4^+ and orgN	[107]				
Cantharellus					Hi		4.3 ± 1.4 (8)	[102]
C. cibarius	NH_4^+ (poor on orgN)			[48]				
Cortinarius					Ho	Medium-fringe	6.8 ± 0.3 (100)	[102]
C. variecolor			orgN	[67]				
Hebeloma					Ho	Short/ medium-fringe	*2.7 ± 1.1 (7)*	[102]
H. cylindrosporum			orgN (but a variable among strains)	[59]				
			ON	[57]				
H. radicosum			NH_4^+	[108]				
Hebeloma sp.			orgN	[108]				
Hydnum					Ho	Medium-fringe	12 (1) cap	[102]
H. repandum	NO_3^- or ON (poor on NH_4^+)			[109]				
Laccaria					Hi	Short	0.5 ± 0.6 (15)	[102]

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
L. lacata	orgN			[110]			3.0 ± 0.4 (3) cap	[111]
	NH_4^+ (poor on orgN)			[112]				
L. bicolor NH_4^+ (poor on NO_3^-, or orgN)				[113]			1.8 (1) cap	[111]
	NH_4^+, NO_3^- (poor on amino acid, good on urea)			[114]				
	NH_4^+ (poor on orgN)			[67]				
Lactarius					Hi	Contact/ Medium-smooth	4.2 ± 0.3 (54)	[102]
							4.3 ± 0.5 (3)	[111]
L. deliciosus	NH_4^+ plus orgN			[98]				
L. rufus	orgN (a variable among strains)			[67]				
Lyophyllus								
L. shimeji			NH_4^+ and orgN	[115]				
Paxillus					Ho	Long	7.1 ± 0.7 (7)	[102]
P. involutus	orgN			[113]				
Scleroderma								
S. citrinum	NH_4^+ or orgN			[112]	Ho	Long		
Suillus					Ho	Long	8.2 ± 0.7 (17)	[102]
S. bovinus			Forest soil	[116]				

ECM fungi	Mycelium growth	Mycorrhization	Fruitbody formation	Ref	Hydrophobicity	Exploration type	$\delta^{15}N$ (‰) (Mean ± SD) (n)	Ref.
S. lutus	NH_4^+ (poor on orgN)			[112]				
S. variegatus	orgN			[113]			5.7 ± 1.1 (4) cap	[111]
Tricholoma					Ho	Medium-fringe	9.3 ± 0.6 (35)	[102]
T. imbricatum	NH_4^+ or NO_3^- or orgN (gained better grwoth in iorgN)			[117]				
T. bakamatsutake	NH_4^+ or orgN (poor on NO_3^-)			[118]				
T. matsutake	NH_4^+ pluse orgN			[119]			16.8 ± 2.3 (15)	[120]
	orgN			[121]				
		orgN (sustaining symbiotic relationship)		[122]				
T. terreum	orgN (gained better growth)			[123]				
Tuber					Hi	Short	15.1 ± 0.6 (9)	[102]
T. sinense	orgN(gained better growth)			[124]				

** NH_4^+, ammonum nitrogen; NO_3^-, nitrate nitrogen; orgN, organic nitrogen. Ho, hydrophobic; Hi, hydrophibic.*

Table 1.
Fungal growth, symbiosis and fruitbody formation observed using different nitrogen sources in edible ectomycorrhizal fungi in combination with the information of hydrophobicity, exploration type and $\delta^{15}N$ of the fruitbodies.

and mycorrhizal formation. In pure culture conditions, most of the studied fungi appeared to favor ammonium N. Some species, namely *Amanita caesarea*, *Cantharellus cibarius*, *Lactarius bicolor*, *Suillus variegatus* were not able to grow nitrate as the sole N source [48, 103, 113].

However, many of the edible ECM fungi, namely *Amanita caesarea* [103, 105], *Cantharellus cibarius* [48], *Cortinarius variecolor* [67], *Paxillus involutus*, *Suillus variegatus* [113], *Tricholoma terreum* [123], and *Tuber sinense* [124] were able to grow on the media containing organic N (protein) as the sole nitrogen. Moreover, some fungi belonging to *Lactarius* genus had limited capacity to utilize protein N [113, 114]. *Hebeloma cylindrosporum* was able to experimentally utilize a wide range of amino acids and other simple (e.g. urea) or complex (e.g. proteins) compounds [6, 59].

The studied forms of N often predominate soil solution and the culturing results might be assumed to hold true in nature. However, it is worth mentioning is that the optimal nitrogen in the mycelium culture does not necessarily reflect the nitrogen preference of the ECM fungus under natural conditions because environmental factors affect. This was shown with *H. cylindrosporum* growing in nature. Wild dikaryotic strains of *H. cylindrosporum* isolated from two different habitat types had different N preferences [6].

Cultivation of edible ECM mushrooms has been successful in cases of two truffles *Tuber melanosporum* Vittad. and *Tuber aestivum* Vittad. They are cultivated commercially around the world [125]. In addition, some success has been achieved with *Lactarius deliciosus* [126, 127] and *Boletus edulis* [128]. Regarding truffle production, it has been suggested that most soils contain enough N to maintain both fungal and tree growth [125]. Similarly, *Lactarius deliciosus* was cultivated experimentally in forest soil, which was observed to meet the demands for fruitbody formation [126]. It has also been demonstrated that the nutritional properties of soil and the forestry history the natural development of ECM mushrooms in forest ecosystems [129]. A productive and diverse ECM mushroom community resembling natural communities developed when abandoned farmland in Mediterranean dry area was forested with *Pinus* sp.

In summary, productive ECM community can grow in natural soils. However, the challenges faced in artificial cultivation has not been solved.

4.2 Nitrogen source requirements for *Tricholoma matsutake* mycelial culture and mycorrhizal synthesis

Tricholoma matsutake is among the most economically valuable mushrooms in the world. Its taxonomy, distribution, ecology, physiology, and cultivation has been studied widely [130]. Here, we summarize the key results linking matsutake ecological characteristics and nutrient requirements focusing on nitrogen.

Matsutake colonizes the roots of its host trees via an ECM association (**Figure 1a** and **b**). It develops an extraradical mycelium in the rhizosphere and in the surrounding soil area. This can be seen as a white rhizosphere area and it corresponds to the mycelium-soil aggregated zone, called a shiro [131] (**Figure 1c** and **d**). Matsutake shiro grows in the form of a concentric or horseshoe-like circle, depending on the rhizosphere conditions, around the host plant at the rate of approximately 10–15 cm per year [131, 132]. The production of matsutake mushrooms changes periodically. Based on our field observations, the part of mycorrhizal root tips is degraded prior to matsutake fruiting. The extraradical mycelium might grow towards new roots and colonizing. Such a hyphal growth strategy indicates that matsutake symbiosis may often need to be renewed and form new mycorrhizas to acquire nutrients (data not published). Among the mycorrhizal associations, such

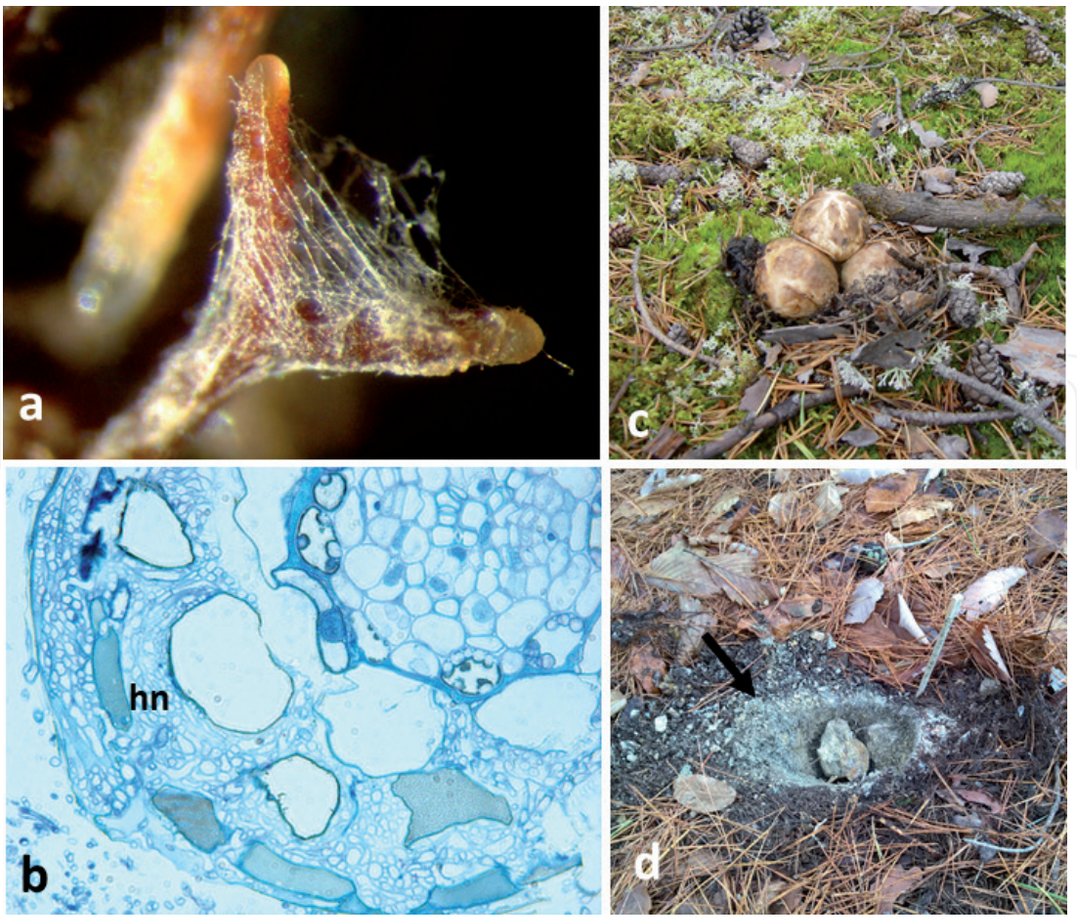


Figure 1.
The ectomycorrhizal edible mushroom—Tricholoma matsutake *(a) the root of* Pinus sylvestris *seedling is colonized by* T. matsutake *fungal mycelium, forms mycorrhizas; (b) the transverse section of ECM root showing the Hartig net (hn) development within the cortex; (c) matsutake mushrooms form in a conifer mixed forest in southern of Finland; (d) the matsutake shiro (arrow) after the mushrooms be harvested (photos were taken by Lu Min Vaario).*

a phenomenon does not seem to be rare. Hortal and colleagues [133] found that the plant had the ability to limit the root tip colonization of the least cooperative symbiont, and therefore, influence the outcome of ECM fungi competition. Such reduction in colonization did not result in a reduction in carbon allocation to the fungus providing the lowest amount of nitrogen.

It is worth noting that decayed mycorrhizal roots together with mycelium-soil aggregated zone might be important organic nutrient sources for matsutake. Recently, the natural abundance of isotopes data showed a very high $\delta^{15}N$ value in *T. matsutake* fruitbodies, which were sampled from Finland and Japan [120]. Matsutake usually grow at B layer of mineral soil [131], such taxa obtain their N could explain for high $\delta^{15}N$ values (see review [102]). More importantly, the high $\delta^{15}N$ value in matsutake is an indicator of organic N uptake from soil because the great variation of ^{15}N content observed among ECM taxa has been reported to be related to the differences in organic N utilization [111]. In addition, a literature study shows that mycorrhizal taxa with proteolytic activities generally show high $\delta^{15}N$ values [67]. Therefore, we conclude that matsutake has a greater proteolytic activity to digest chemically complex ^{15}N-enriched organic matter in soil during matsutake fruitbody development.

In addition to proteases, matsutake produces organic matter degradation enzymes such as acid proteinase [134, 135] and β-glucosidase [136]. Relatively high enzyme activities, β-glucosidase and xylosidase, were detected from matsutake cultures in vitro and in shiro soil [137, 138]. The genome of *T. matsutake* encodes

two GH7 cellobiohydrolases [88], which is in agreement with its known facultative saprotrophic activity [136, 138]. However, no further evidence of any strong saprotrophic characteristics of matsutake was found. It could be speculated that these ECM fungi produce certain levels of carbohydrase, not to fully degrade organic matter to access C but N. Kawai and Abe [121] reported that dried beer yeast, corn steep liquor, casein hydrolysate, and polypeptone were good N sources for matsutake mycelium culture whereas nitrate was not. Dry beer yeast (Ebios, Asahi Beer Inc., Tokyo, Japan), as the sole N source, showed promising matsutake mycelium growth and as well mycorrhizal formation [139] (personal communication with Dr. A. Yamada).

Several agar media such as MMN, MNC, Hamada containing both inorganic and organic N are widely used to culture the mycelium of *T. matsutake* [119, 140]. However, the question whether matsutake prefers organic nitrogen is worth of considering. Usually, more inorganic N than organic N is present in the soil top layer. Some studies suggested that increased N deposition could reduce fruitbody production [141]. Nohrstedt [142] reported a 30% decrease in sporocarp production by *Cantharellus cibarius* in a central Swedish pine forest after the application of 150 kg N ha^{-1} ammonium nitrate. The presence of nitrate ions has been shown to have negative effects on the development of some ECM fungi both in vitro and in soil [143, 144]. Removal of the litter layer has been considered an important method to improve the productivity of matsutake in many Asian countries [145]. It has also been shown that the removal of the upper organic soil layers of the forest floor can improve the sporocarp production of some other ECM fungi [146, 147]. The explanation might be that competition with other microbes diminishes. Litter and organic soil provide carbon and nutrients for microbes, especially for saprotrophic fungi that would compete with *T. matsutake* in the shiro [148].

4.3 Research prospects

Cultivation of ectomycorrhizal mushrooms is still facing many challenges. Although some species of ECM fungi can form the primordium of fruiting bodies on several media, they usually do not develop further into mature fruiting bodies. So far, the most successful efforts have been carried out with the mycorrhizal plants growing in soil. Soil nutrients and soil microbial communities together with climatic factors have shown to affect significantly the persistence of ectomycorrhizas in outplanted inoculated plants, and further, the successful fruiting. The observed suppression of many mycorrhizal mushrooms has been linked to indirect effects of air pollution, in particular to increases in nitrogen deposition accumulating into litter and humus [149, 150]. A thorough understanding of the ecological and environmental factors regulating the ECM fungal species is a prerequisite for their cultivation.

Ectomycorrhizal fungi colonize the roots of their host plants and improve plants' access to nutrients, especially nitrogen. In exchange, host plants deliver a significant portion of their photosynthesized carbon to the ECM fungi. However, we need more accurate understanding of the ECM fungi mediated C and N movement within forest ecosystems. ECM fungi may follow a similar pattern with the amount of C delivered being related to the amount of N sourced by the fungus [77, 151], although this is still controversial [133]. Production of ECM mushrooms do need a balanced nutrient either assimilating by ECM fungi or by other soil microbial.

It has been suggested that the growth of ECM fungi and the formation of mycorrhizas are promoted by certain mycorrhizosphere bacteria, termed 'mycorrhizal helper bacteria' [152]. Some mycorrhizal fungi-associated bacteria are also known to fix nitrogen [153, 154]. However, there is still no evidence that the fungus would

directly benefit from its associated bacteria. Sporocarps of *Cantharellus cibarius* contain large amount of bacteria, in particular fluorescent *Pseudomonas* [155]. Some species of bacteria such as *Streptomyces* spp., *Paenibacillus* spp. and *Bacillales* spp. were isolated from the mycorrhizal root tips and fruitbodies of *T. matsutake* as well [156–158]. Otherwise, the information about mycorrhizas-associated bacteria and their effect on the nutrient uptake of ECM fungi is limited. These studies, however, hint that the production of ectomycorrhizal mushrooms may require teamwork to obtain enough nutrients from the environment.

In conclusion, ECM fungi play an important role in the nutrient cycle of forest ecosystems, especially on mediating C and N movement. A better understanding of the nitrogen status of the habitat of ECM fungi, nutrients movements within the ecosystems, as well as the ECM fungal hyphal structures should be the first step for cultivation of ECM edible mushrooms. The methodological advances in these areas in combination with forest management may allow the successful establishment of commercial plantations and production of edible ECM mushrooms in forests.

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