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Ectomycorrhizal fungi and the enzymatic liberation of nitrogen from soil organic matter: why evolutionary history matters

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Summary

The view that ectomycorrhizal (ECM) fungi commonly participate in the enzymatic liberation of N from soil organic matter (SOM) has recently been invoked as a key mechanism governing the biogeochemical cycles of forest ecosystems. Here, we provide evidence that not all evolutionary lineages of ECM have retained the genetic potential to produce extracellular enzymes that degrade SOM, calling into question the ubiquity of the proposed mechanism. Further, we discuss several untested conditions that must be empirically validated before it is certain that any lineage of ECM fungi actively expresses extracellular enzymes in order to degrade SOM and transfer N contained therein to its host plant.

Key words: ectomycorrhiza, evolution, extracellular enzymes, nitrogen (N), soil organic matter (SOM), symbioses.

I. Introduction

In many terrestrial ecosystems, nitrogen (N) mineralization is often insufficient to account for annual plant N demand (Näsholm *et al.*, 2009). To rectify this disparity, organic soil N has been hypothesized to be an important component of plant N supply (Neff *et al.*, 2003).

Ectomycorrhizal (ECM) fungi play a significant role in provisioning plants with N. Through the production of prolific hyphae, these fungi compose one-third of microbial biomass in boreal and temperate ecosystems (Högberg & Högberg, 2002); ECM fungi greatly increase the volume of soil exploited by fine roots and hence increase inorganic N uptake by plants (Smith & Read, 2008). Additionally, ECM fungi assimilate amino acids and amino sugars from soil solution (Lilleskov *et al.*, 2002; Smith & Read, 2008), degrade proteins (Bending & Read, 1996; Read & Perez-Moreno, 2003), and transfer the N contained therein to their plant hosts (Abuzinadah & Read, 1996a; Näsholm *et al.*, 2009).

However, *c*. 95% of soil N is complexed in the end products of plant and microbial decay, collectively known as soil organic matter (SOM; Schulten & Schnitzer, 1998; Rillig *et al.*, 2007). While saprotrophic fungi and some bacteria possess the capacity to metabolize SOM, the idea that ECM fungi obtain N bound in SOM has recently become generalized in a wide body of literature (Shah *et al.*, 2015; Cheeke *et al.*, 2016; Averill & Hawkes, 2016; Trap *et al.*, 2016). This purported ECM physiology has significant implications for understanding soil biogeochemical cycles (Orwin *et al.*, 2011; Averill *et al.*, 2014), as well as models of plant NPP. Acquisition of N from SOM has been postulated to provide plants with an additional source of growth-limiting N, thereby allowing sustained growth under elevated atmospheric CO_2 (Terrer *et al.*, 2016). The aforementioned studies argue their results arise, at least in part, from the physiological capability of ECM fungi to use lignocellulolytic enzymes that depolymerize SOM and transfer the N therein to the host plant. However, this generalization ignores the fact that ECM fungi have independently and differentially evolved from saprotrophic ancestors dozens of times (Hibbett *et al*., 2000), causing the degree to which they have retained genes with saprotrophic function to dramatically differ among lineages (Kohler *et al.*, 2015). The unique evolutionary history of each ECM lineage seriously draws into question the assumption that all ECM function similarly to provide host plants with N bound in SOM.

Understanding the capacity for ECM fungi to obtain N from SOM and transfer it to their plant host requires the empirical validation of several conditions outlined in Fig. 1. Foremost, the extent to which ECM fungi metabolize SOM is first contingent on whether genes encoding lignocellulolytic enzymes (*i.e.*, glycoside hydrolases, class II fungal peroxidases, glyoxyl oxidases, and phenol oxidases) were retained during their evolutionary history and are deployed when in symbiosis with plant roots. By critically reviewing pertinent studies and their bearing on

the conditions presented in Fig. 1, we conclude that, to the best of our knowledge, direct evidence establishing that all these conditions simultaneously occur for any ECM fungi is presently absent from the literature. Finally, we discuss recent biogeochemical observations with respect to the implications derived from our conclusions.

II. Have ECM fungi retained genes with lignocellulolytic potential from saprotrophic ancestors?

ECM fungi evolved primarily in the Basidiomycetes, but independent ECM lineages also appear in the Ascomycota (Smith & Read, 2008). The evolution of genes encoding lignin and manganese peroxidases, however, appear to have primarily evolved in the Basidiomycota (Floudas *et al.,* 2012). Accordingly, Ascomycete ECM fungi, such as the widespread *Cenoccoccum geophilum*, are unlikely to possess the genetic potential to depolymerize SOM.

Although the ancestor to the Agaricomycete clade has been reconstructed as a white rot saprotroph (Hibbett *et al.,* 2000; Floudas *et al.,* 2012), the evolution of the ECM lifestyle was thought to involve large losses of genes mediating the decay of lignocellulose and phenolic compounds in SOM (Martin *et al.,* 2008; Plett & Martin, 2011; Wolfe *et al.,* 2012). Recently, however, numerous copies of genes potentially mediating the decay of SOM were observed in some lineages of ECM fungi (Bödeker *et al.,* 2009; Kohler *et al.,* 2015; Fig. 2). In fact, the largest survey of ECM fungal genomes to date revealed that some ECM possess genes encoding class II peroxidases, glyoxal oxidases, cellobiohydrolases, laccases, and other enzymes which, when present in the genomes of white and brown rot fungi, mediate the saprotrophic decay of plant and microbial detritus as well as SOM (Kohler *et al.,* 2015). Available evidence shows that ectomycorrhizal genomes have fewer lignocellulolytic genes than do their saprotrophic ancestors (Martin *et al.,* 2016). The occurrence of these genes has led some to speculate that ECM fungi actively transcribe them into enzymes that depolymerize complex organic macromolecules in SOM, thereby providing plants access to the large pools of N previously theorized to be unavailable for plant uptake (Bödeker *et al.,* 2014; Lindahl & Tunlid, 2015).

Importantly, the occurrence of genes with saprotrophic function varies widely across lineages of ECM fungi (Fig. 2). For instance, *Amanita muscaria* evolved within a clade of brown rot saprotrophs and consequently has lost the genetic capacity to depolymerize organic matter (Wolfe *et al.,* 2012; Kohler *et al.,* 2015). Similarly, *Laccaria bicolor* has lost most genes

encoding enzymes that act on crystalline cellulose and lignin, although its genome does contain 11 copies of lytic polysaccharide monooxygenases (LPMO; Martin *et al.*, 2008; Kohler *et al.*, 2015). Ectomycorrhizal taxa in the well-known Boletales clade occur within a paraphyletic group of brown rot fungi; these ECM taxa generally lack the genetic potential to degrade the polyphenolic and polysaccharide components of plant cell wall, microbial residues, as well as analogous compounds in SOM (Kohler *et al.*, 2015; Fig. 2). For the Boletales, it appears that parallel losses of genes mediating saprotrophic decay occurred in each of the three independent originations of a mutualistic lifestyle (Kohler *et al.*, 2015).

By contrast, *Hebeloma cylindrosporum* evolved from a white rot ancestor that uses class II fungal peroxidases to oxidize polyphenolic compounds in SOM (Kohler *et al.*, 2015). *H. cylindrosporum* has retained 3 copies of class II peroxidases, as well as 3 LPMO copies (Kohler *et al.*, 2015; Fig 2.). Lastly, *Cortinarius glaucopus* has retained the greatest known number of genes with putative saprotrophic function, including 11 Mn-peroxidases derived from white rot saprotrophic ancestors (Bödeker *et al.*, 2014; Martin *et al.*, 2016). Clearly, the genetic potential to decay SOM varies widely across lineages of ECM fungi, making broad generalizations about the role of these organisms as agents of litter and SOM metabolism tenuous at best.

As each of the aforementioned lineages evolved into ECM fungi, hypothetical selective and drift processes governed the retention or loss of genes involved in the depolymerization of SOM. If ECM fungi evolved under conditions in which the fungi or host plants were consistently N limited, there may have been selective pressure to maintain energetically expensive lignocellulolytic genes that mediate the release of N from SOM. Further, because the ECM lifestyle evolved repeatedly over a relatively large span of evolutionary time, it would be unlikely that each of the dozens of independent transitions to ECM symbiosis resulted in the same whole-genome alterations (*i.e.*, loss of genes with saprotrophic function). This phenomenon has some precedent in the fungal symbionts of ambrosia beetles, in which each of the multiple origins of ambrosia fungi experienced different patterns of gene loss or gain (Cassar & Blackwell, 1996). In sum, because ECM fungi have lost genes with saprotrophic function over their evolutionary history in a differential manner (Martin *et al.*, 2016), we should not expect that they represent a single functional group that uniformly provides host plants with N from SOM.

III. Are genes with saprotrophic function expressed by ECM fungi when in symbiosis?

In cases where ECM taxa have retained genes encoding enzymes that mediate SOM decay, it remains unclear if these genes are actively transcribed while forming mycorrhiza **(**Fig. 1). Ecological predictions based on the results of whole-genome sequencing suggest that ECM fungi retaining the largest numbers of class II fungal peroxidase genes should have the greatest physiological ability to oxidize the polyphenolic compounds in SOM (Kohler *et al.,* 2015). However, available evidence does not support this hypothesis when it has been tested in culture. Rather, multiple studies have found that the number of Mn-peroxidase genes present in ECM genomes did not predict enzyme activity when grown on SOM extracts (Shah *et al.,* 2015) or leaf litter (Talbot *et al.,* 2015).

Insights into the ability of ECM to synthesize lignocellulolytic enzymes and alter the biochemistry of SOM and plant cell wall in culture are intriguing (Rineau *et al.,* 2012; Talbot *et al.,* 2015; Shah *et al.,* 2015); but because transcriptional profiles of ECM fungi vary depending upon whether the fungi are free-living or actively forming mycorrhiza (Martin *et al.,* 2008; Kohler *et al.,* 2015; Liao *et al.,* 2016), these studies cannot conclusively demonstrate that identical physiology occurs when forming mycorrhiza. In fact, although *L. bicolor* has a limited genetic capacity to degrade plant cell wall, it did not transcribe these genes while forming mycorrhiza (Martin *et al.,* 2008). Accordingly, there are few definitive studies demonstrating that ECM fungi express genes encoding saprotrophic enzymes while in symbiotic association with their plant hosts. In an important exception, Bödeker and colleagues (2014) observed that ECM fungi in the genus *Cortinarius* express high levels of Mn-peroxidase in boreal forest soils.

Finally, if genes with saprotrophic potential are expressed under field conditions, it is likely that their expression is context-dependent, determined by a myriad of ecological and edaphic factors including pH, the availability of inorganic N and organic N in soil solution. This phenomenon is well known for saprotrophic fungi (Sinsabaugh *et al.,* 2010; Edwards *et al.,* 2011) and has now been demonstrated for ECM fungi in the field (Bödeker *et al.,* 2014). For example, when NH_4^+ is added to soils in the field, the expression of ECM genes with lignocellulolytic capacity are downregulated (Bödeker *et al.,* 2014). Extrapolations from these patterns suggest that ECM fungi deploying Mn-peroxidases and other energetically expensive lignocellulolytic enzymes do so only when they and or their plant hosts are N limited. Resolving

the abiotic conditions and nutritional status of the host plant, as well as symbiont in which these ECM genes reside, will add significant insight to our understanding of this phenomenon.

IV. Do transcribed enzymes operate to obtain N from SOM?

Classic studies demonstrated that certain ECM fungi can obtain N from various organic sources (Abuzinadah & Read 1986, 1988; Smith & Read, 2008). Below, we clarify why previous work has not definitively resolved the ability for ECM fungi to obtain N from SOM. Evidence that ECM obtain organic N and transfer it to their host plants comes primarily from studies using pure protein or amino acids as an organic N source (Abunzinadah & Read, 1986; Lilleskov *et al.*,2002). However, most protein in soil is complexed with SOM or mineral surfaces (Nannipieri & Paul, 2009; Rillig *et al.,* 2007), and moreover the macromolecular structure of SOM results in amino acids, amino sugars, and protein being both physically and chemically resistant to enzymes that degrade peptides (Rillig *et al.,* 2007). Thus, studies using pure protein as an N source are unlikely to be ecologically realistic (Jones *et al.,* 2005; Nannipieri & Paul, 2009). Recognizing these challenges, Bending & Read (1996) provided N to ECM fungi as a protein-polyphenol complex in pure culture, and observed that ECM fungi displayed significantly reduced uptake of organic N.

An additional level of uncertainty associated with the function of saprotrophic genes in ECM fungi arises from the fact that the enzymes they encode may have alternative functions. ECM transcribing laccase genes, thereby producing phenol oxidase enzymes, have been suggested as important to SOM decay (Talbot & Treseder, 2010; Shah *et al.,* 2015). However, laccase genes can transcribe both extra- and intracellular enzymes, the latter of which is involved with rigidifying fungal cell walls, producing melanin, and detoxifying cells (Kues & Ruhl, 2011). Apart from Mn- and lignin peroxidases whose substrates are inherently extracellular (Baldrian, 2006), the occurrence and expression of fungal laccase genes need not imply that ECM express them to operate extracellularly on SOM.

Finally, it has been assumed that the oxidative potential of the enzymes expressed by ECM fungi is equivalent to those produced by saprotrophic fungi (Bödeker *et al.,* 2014). Nonetheless, genes annotated as Mn-peroxidases in ECM fungi, including *H. cylindrosporum, C. glaucopus* and *P. croceum* were described as ‘atypical’ (Kohler *et al.,* 2015; Shah *et al.,* 2015; Martin *et al.,* 2016), suggesting that the conformation of enzymes transcribed from these genes

may not allow for the oxidation of lignin and polyphenols. Presently, we simply do not understand the effectiveness of the array of enzymes produced by ECM to oxidize or hydrolyze the wide range of organic bonds in SOM.

V. Is the organic N derived from SOM transferred to the plant host?

Finally, if some ECM taxa obtain N from SOM, they may not transfer this N to their plant hosts (Fig. 1). For example, Abuzinadah *et al*. (1986) **[Author, Abuzinadah & Read (1986a) has been altered to Abuzinadah *et al.* (1986) for consistency with the References. Please check that this is correct.]** noted that *Paxillus involutus* obtained N from pure protein, but in so doing sequestered high quantities of this N into its mycelium. This may be a general phenomenon, because current evidence for ECM fungi expressing saprotrophic enzymes in the environment occurs in boreal forests (Bödeker *et al.,* 2014); other studies in the boreal forest reveal that ECM fungi transfer only small amounts of organic N to their plant hosts (Näsholm *et al.,* 2013). In fact, modeling efforts in boreal forest ecosystems suggest that increasing atmospheric CO_2, in direct contrast to Terrer *et al.* (2016), will exacerbate plant N limitation through increases in the biomass and N content of fungal mycelium, even as plants allocate greater amounts of photosynthate belowground (Näsholm *et al.,* 2013; Franklin *et al.,* 2014). We conclude that the role of ECM in enzymatically liberating N from SOM and transferring it to host plants is an open question, and, at present, no *direct evidence* has been gathered to demonstrate that ECM enhance plant N supply by accessing N in SOM.

VI. Concluding remarks

The rationale we present here highlights the significant complications invoked by the broad assumption that all ECM fungi liberate N from SOM. Until additional experimental evidence accumulates, it is ambiguous, at best, whether ECM fungi can provision their host plants with N from SOM. There is, however, an urgent need to test this possibility under field conditions, as multiple studies suggest this mechanism holds significant implications for the construction of accurate coupled climate-biogeochemical models (Orwin *et al.,* 2011; Averill *et al.,* 2014; Terrer *et al.,* 2016).

We note that the intriguing patterns observed in some recent biogeochemical models likely occur through a plurality of belowground interactions, including the physical and

biochemical activity of ECM fungi on their soil surroundings. ECM may contribute to SOM dynamics through the accumulation of hyphae to exploit soil volume, which can vary substantially in architecture and biomass (Clemmensen *et al.*, 2015); further, hyphal traits such as melanization may render some hyphae more resistant to decay than others (Fernandez & Koide, 2014). Accumulation of hyphae, and the greater functional exploitation of soil volume for inorganic and simple organic N compounds, is distinct from the idea that certain ECM fungi biochemically alter SOM through the exudation of extracellular lignocellulolytic enzymes. As we have discussed, lineages of ECM fungi that have retained genes with lignocellulolytic capacity are more likely to depolymerize SOM than others. Decay-resistant hyphae and enzymatic activities need not be mutually exclusive, and it is plausible that certain fungal communities contribute to SOM dynamics more than others. Finally, given current knowledge of the turnover of ECM communities across space (Talbot *et al.*, 2014), it is unlikely that ECM lineages with the potential to operate on SOM are distributed uniformly across ecosystems. Therefore, improved understanding of the biogeographic distribution and enzymatic physiology of different lineages of ECM fungi will instruct ecologically realistic models of C and N cycling across ecosystems.

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Fig. 1 Set of minimum conditions necessary for the liberation of organic nitrogen (N) from soil organic matter (SOM) by ectomycorrhizal fungi (ECM). See full text for explanation.

Fig. 2 Agaricomycete gene copy number for enzymes acting on organic matter, (a) class II peroxidases and (b) laccase gene copy number. Numbers at tips represent the number of genes observed, numbers at nodes represent reconstructed gene copy number. Question marks indicate uncertain estimates. Ecology of taxa are color coded as follows: ectomycorrhizal (ECM) fungi, green; White Rot, yellow; Brown Rot, brown; all others, purple. Legend: average gene copy numbers for Agaricomycotina: blue, at or above average; yellow or orange, below average; red, no copies of the gene family. Nodes with question marks indicate uncertain estimates. Black star, independent ECM origination. Modified with kind permission from Kohler *et al.* (2015).