A widespread mechanism in ectomycorrhizal fungi to access nitrogen from mineral-associated proteins

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Summary

A large fraction of nitrogen (N) in forest soils is present in mineral-associated proteinaceous compounds. The strong association between proteins and minerals limits microbial accessibility to this source, which is a relatively stable reservoir of soil N. We have shown that the ectomycorrhizal (ECM) fungus Paxillus involutus can acquire N from iron oxide-associated proteins. Using tightly controlled isotopic, spectroscopic and chromatographic experiments, we demonstrated that the capacity to access N from iron oxide-associated bovine serum albumin (BSA) is shared with the ECM fungi Hebeloma cylindrosporum and Piloderma olivaceum. Despite differences in evolutionary history, growth rates, exploration types and the decomposition mechanisms of organic matter, their N acquisition mechanisms were similar to those described for P. involutus. The fungi released N from mineral-associated BSA by direct action of extracellular aspartic proteases on the mineral-associated BSA, without initial desorption of the protein. Hydrolysis was suppressed by the adsorption of proteases to minerals, but this adverse effect was counteracted by the secretion of compounds that conditioned the mineral surface. These data suggest that the enzymatic exudate-driven mechanism to access N from mineral-associated proteins is found in ECM fungi of multiple lineages and exploration types.

Introduction

Ectomycorrhizal (ECM) fungal symbionts play a critical role in providing N to plants in temperate and boreal forests (Hobbie and Högberg, 2012). They supply host trees with soil-derived N, which in turn provide the fungi with sugars (Smith and Read, 2008). A large part of soil N is present in organic form, particularly as proteinaceous compounds that constitute 30%–50% of the total soil organic N stock (Nannipieri and Paul, 2009; Leinweber et al., 2013). Organic N molecules are associated with complex mixtures of plant- and microbe-derived compounds that comprise the soil organic matter (SOM). The availability of soil organic N is further restricted by the association of 20%–50% of total SOM in boreal forests with reactive soil minerals (Kramer and Chadwick, 2018). In particular, proteins bind strongly to mineral surfaces (Kleber et al., 2007; Mitnner et al., 2012); these associations limit enzymatic accessibility to substrate proteins (Chevallier et al., 2003). It is commonly thought that the proteins must be desorbed from the mineral surface before they become available for enzyme degradation (Mikutta et al., 2007; Schimel and Schaeffer, 2012; Jilling et al., 2018). For this reason, mineral-associated proteins are rarely considered as bioavailable N for soil microorganisms (Jilling et al., 2018).

A recent study by our group challenged this view by showing that the ECM fungus Paxillus involutus can assimilate N from iron oxide-associated bovine serum albumin (BSA) by hydrolyzing the protein directly at the mineral surface without its initial desorption (Wang et al., 2020). The extent of proteolysis of mineral-associated BSA was lower than that of soluble BSA, which was partly attributed to the adsorption of fungal proteases to vacant surface sites of iron oxides. Notably, this adsorption was counteracted by low-molecular-weight (LMW) exudates secreted by the fungus. Some of these compounds are adsorbed to mineral surfaces (Wang et al., 2017), suggesting that they might decrease the binding of the proteases to minerals, thereby enhancing proteolysis. In an accompanying paper, the...
proteolysis of BSA associated with iron oxide was studied in more detail using a fungal aspartic protease isolated from the soil ascomycete Rhizopus sp. (Tian et al., 2020). For this enzyme, the initial rate of proteolysis of BSA associated with iron oxide was similar to that of soluble BSA, but the rate slowed more rapidly at the mineral surface than in solution. This inhibitory effect was counteracted by the adsorption of phosphate to the mineral surface with high affinity (Tian et al., 2020). Therefore, experiments with the Rhizopus protease support the view that some fungal aspartic proteases can directly hydrolyze iron oxide-associated proteins, with their reaction rates strongly regulated by environmental conditions, including the prevalence of strong ligands that counteract the adsorption of proteases on minerals.

An important step for evaluating the significance of this pathway in ECM fungi is to test for its presence in other species. Therefore, in this study, we evaluated the capacity and mechanisms by which the ECM fungi Hebeloma cylindrosporum and Piloderma olivaceum can assimilate N from iron oxide-associated BSA. Hebeloma cylindrosporum and P. olivaceum are found in two different clades that are well separated from P. involutus. These species represent three different origins of ECM symbiosis. They have evolved from saprotrophic ancestors with diverse decomposition mechanisms, including the two major lignocellulose decomposition mechanisms identified in wood-decaying fungi: the enzymatic mechanism of white-rot fungi and the non-enzymatic, Fenton-based mechanism of brown-rot fungi (Kohler et al., 2015). Hebeloma cylindrosporum belongs to the Agaricales clade and is nested in an assemblage of white-rot wood decayers. Piloderma olivaceum is nested within the Atheliales clade, which includes white-rot saprotrophs (Sulistyo et al., 2021), whilst P. involutus is nested within a paraphyletic assemblage of brown-rot wood decayers in the Boletales clade (Kohler et al., 2015).

Although ECM fungi have lost several genes for decaying SOM that were present in their saprotrophic precursors, it has been reported that H. cylindrosporum, P. olivaceum and P. involutus can oxidize organic matter to some extent when assimilating N from complex SOM (Shah et al., 2016). The genome of H. cylindrosporum contains three class II peroxidases and P. olivaceum contains one generic peroxidase whereas P. involutus lack such enzymes (Kohler et al., 2015). It has been shown that P. involutus oxidizes SOM using hydroxyl radicals (•OH) generated by Fenton chemistry similar to the mechanism of brown-rot wood decay (Rineau et al. 2012; Op De Beeck et al., 2018). Hebeloma cylindrosporum and P. olivaceum have a large number of genes encoding extracellular endo- and exopeptidases; these gene sets are similar but not identical to those of P. involutus (Kohler et al., 2015). Moreover, these three fungi have different growth strategies, i.e., exploration types. H. cylindrosporum and P. olivaceum exhibit short/medium-fringe and short-distance exploration types respectively, with slow-growing mycelia; in contrast, P. involutus is a long-distance, fast-growing species with high biomass (Agerer, 2006). The fungi also differ in the types of environment in which they are found. P. olivaceum is commonly associated with old forest stands and can be found in both organic and mineral soil horizons (Rosling et al., 2003; Herrmann and Buscot, 2007), whereas H. cylindrosporum is considered to be a pioneer species that thrives in newly established forests (Marmeisse et al., 2004).

An additional reason for selecting H. cylindrosporum and P. olivaceum is that they can be grown in the same axenic system as P. involutus to study their decomposition of organic matter (Shah et al., 2016). This system is compatible with the detailed isotopic, spectroscopic and chromatographic measurements required for quantifying and analysing the mechanism for N acquisition from mineral-associated proteins (Wang et al., 2020). Using this system, we addressed two primary research questions: (1) Is the capacity to acquire N from iron oxide-associated proteins found in ECM fungi with different evolutionary origins, SOM-decomposition mechanisms, exploration types and ecologies? (2) If so, do the fungi use a common mechanism involving direct hydrolysis of mineral-associated proteins?

Results

N assimilation from mineral-associated BSA

The iron oxides used in our experiments were ferrihydrite and goethite, which are abundant in forest soils and exhibit differences in specific surface area and redox potential, which may influence the stabilisation of adsorbed organic matter (Krumina et al., 2017; Hall et al., 2018). Both were investigated to assess the general nature of the results in light of the research questions. To vary the concentrations of vacant mineral sites, the experiments were conducted using iron oxides with low (0.7 mg m⁻²) and high (1.4 mg m⁻²) BSA surface concentrations. Negligible amount of mineral-associated BSA was released into the aqueous phase from the mineral surfaces during a fungus-absent incubation for five weeks (Fig. S1).

The biomass carbon (C) increased approximately twofold for both H. cylindrosporum and P. olivaceum when incubated for five weeks on the medium containing ferrihydrite- and goethite-associated BSA (Table 1). For ferrihydrite, but not goethite, the biomass C was higher in medium containing high than low BSA surface
Along with growth, the $^{15}$N atom % of the isotope-labelled mycelia decreased, suggesting an uptake of N from the non-labelled mineral-associated BSA (Fig. S2). Using isotope mixing models (Wang et al., 2020), we estimated that the fungi assimilated 26–45 and 37–57% N from the ferrihydrite- and goethite-associated BSA respectively (Fig. 1). Hebeloma cylindrosporum tended to assimilate more N than P. olivaceum, but the difference was not significant ($P > 0.05$). More N was assimilated from iron oxide with high BSA surface concentrations, although the difference was significant only for ferrihydrite (Fig. 1). During incubation, the N content of the mineral-associated BSA decreased, with depletion roughly equal to the amount of assimilated N (Fig. S3). The $^{13}$C atom % of the isotope-labelled mycelia also decreased (Fig. S2d). According to the isotope mixing models, 4 ± 1% and 11 ± 6% of mineral-associated BSA C were detected in the mycelia of P. olivaceum and H. cylindrosporum respectively, indicating that a part of BSA C was acquired and used by both fungi to synthesize cellular compounds. These values were substantially lower than the assimilated fractions of BSA N (cf. Fig. 1 where 26%–57% of mineral-associated BSA N was assimilated), suggesting that the fungi recycled less C than N from the hydrolysed BSA.

For both fungi, the N acquired from the iron oxide-associated BSA was substantially lower than that from soluble BSA. The fractions of N assimilated from medium with soluble BSA (100 mg L$^{-1}$; the amount was equal to that in the medium containing ferrihydrite or goethite-associated BSA at 0.7 mg m$^{-2}$ and 1.4 mg m$^{-2}$ respectively) was 87 ± 15% for P. olivaceum and 91 ± 33% for H. cylindrosporum (mean values ±1 SD, $n = 4$).

The compounds adsorbed by the iron oxides were desorbed from the mineral particles using a strong phosphate buffer, then analysed by size exclusion chromatography (SEC) (Wang et al., 2020). Before fungus inoculation (i.e., 0 day), the desorbed fractions showed

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**Table 1.** N acquisition, secretion of mineral surface-reactive compounds and iron oxide dissolution by P. olivaceum, H. cylindrosporum and P. involutus when grown on iron oxide-associated BSA as N source. Data for P. involutus were retrieved from Wang et al. (2020).

| Phylogeny and growth strategy | Piloderma olivaceum | Hebeloma cylindrosporum | Paxillus involutus |
|-------------------------------|---------------------|------------------------|-------------------|
| **Phylogeny and growth strategy** | Atheliales | Agaricales | Boletales |
| Clade | Short-distance | Short/medium-fringe | Long-distance |
| Exploration type | Slow | Slow | Fast |
| Growth conditions | Incubation time (days) | 35 | 35 | 8 |
| Initial biomass C (mg per petri dish) | 2.2 | 6.5 | 3.5 |
| Final biomass C (mg per petri dish) | 4.1 | 11.9 | 7.6 |
| Glucose consumption (% of added) | 98.5 | 99.7 | 86.0 |
| **Assimilation of N** | Mineral-associated BSA, 0.7 mg m$^{-2}$ (% of added) | 32 | 40 | 9 |
| Mineral-associated BSA, 1.4 mg m$^{-2}$ (% of added) | 42 | 51 | 32 |
| Free BSA, 100 mg L$^{-1}$ (% of added) | 87 | 92 | 78 |
| **Proteolytic capacity** | Proteolytic activity | 8 | 5 | 1 |
| Inhibition with pepstatin A (remaining activity,% of total) | 4 | 5 | 10 |
| **Mineral surface-reactive compounds** | Adsorbed C (% of new biomass C) | 3.7 | 1.9 | 1.3 |
| Adsorbed N (% of new biomass N) | 4.9 | 4.5 | 0.8 |
| C/N ratio | 14 | 18 | 51 |
| **Chemical composition** | Carboxylates, carbohydrates, peptides | Carboxylates, carbohydrates, peptides | Carboxylates, phenolics, carbohydrates |
| Iron oxide dissolution | Fe$^{2+}$ from ferrihydrite (mg g$^{-1}$ ferrihydrite) | 1.5 | 2.8 | 5.1 |
| Fe$^{2+}$ from goethite (mg g$^{-1}$ goethite) | 0.2 | 0.3 | 0.4 |

*aAverage values for experiments with ferrihydrite- and goethite-associated BSA.

*bProteolytic activity in culture filtrates after growth on soluble BSA. P. olivaceum and H. cylindrosporum were incubated for 35 days and P. involutus for 9 days. Activity was analysed using BSA as the substrate. Activity in the filtrates of P. involutus was set to 1. Values were not adjusted for differences in incubation times.

*cAverage values adsorbed by goethite. Adsorption of fungal-derived C and N was slightly higher on ferrihydrite than on goethite.

*dInferred from IR spectroscopy of secreted compounds adsorbed on ferrihydrite or goethite after growth on media containing mineral particles without associated BSA.

*eAverage Fe$^{2+}$ generated by reductive dissolution after growth on ferrihydrite-associated or goethite-associated BSA.
the presence of a dominant compound with an estimated molecular weight (MW) of ~75 kDa (Fig. 2A), which is close to the MW of BSA (Folta-Stogniew and Williams, 1999). After fungal growth, the intensity of the BSA peak decreased significantly, while two broad peaks centered at ~29 kDa and ~10 kDa appeared, indicating the hydrolysis of mineral-associated BSA and the formation and subsequent adsorption of LMW proteolytic products. SEC indicated that *H. cylindrosporum* hydrolyzed a greater amount of the iron oxide-associated BSA than did *P. olivaceum* (Fig. 2A).

Infrared (IR) spectroscopy of the mineral particles verified the hydrolysis of mineral-associated BSA and the formation of proteolytic products (Fig. 2B). After fungus incubation, the intensities of the amide I (~1658 cm\(^{-1}\)) and amide II (~1536 cm\(^{-1}\)) bands characteristic of the proteins decreased. The intensities around 1600 and c. 1400 cm\(^{-1}\) decreased to a lesser extent than the amide bands or were slightly increased after fungal growth. These data suggested that carboxylate-bearing compounds were formed during proteolysis and were adsorbed on the mineral particles (Tian et al., 2020).

**Secretion of mineral surface-reactive exudates and mineral dissolution**

Both species secreted substantial amounts of mineral surface-reactive exudates when grown in media containing iron oxide mineral particles with or without associated BSA (Fig. 3A). Based on isotope analyses, the amounts of fungus-derived C and N adsorbed on goethite corresponded to 3.7% and 4.9% and 1.9% and 4.5% of the newly formed biomass C and N for *P. olivaceum* and *H. cylindrosporum* respectively (Table 1). The adsorption of fungus-derived C and N was slightly higher on ferricydrite than on goethite (Fig. 3A; Fig. S4A). The C/N ratios of the mineral surface-reactive exudates varied between species, mineral types and BSA surface concentrations, indicating large variations in chemical composition (Fig. S4B). The fungi also secreted mineral surface-reactive exudates when grown on medium containing soluble BSA [i.e., without mineral particles; 0.046 ± 0.002 and 0.064 ± 0.001 mg C per Petri dish for *P. olivaceum* and *H. cylindrosporum* respectively; mean values ±1 SD (n = 3)]. These levels were substantially lower than exudate production in media containing mineral-associated BSA (Fig. 3A).

The chemical composition of the mineral surface-reactive exudates produced by the fungi was analysed by IR spectroscopy. To avoid interference from bands associated with BSA and its proteolytic products, fungi were grown on media with mineral particles but lacking BSA. The IR spectra showed bands at ~1600 cm\(^{-1}\) and 1400 cm\(^{-1}\), suggesting the presence of compounds containing carboxylate moieties (Fig. 3B). The exudates contained other functional groups, as indicated by the IR peaks centered at 1660, 1540 and 1070 cm\(^{-1}\). The bands at 1660 and 1540 cm\(^{-1}\) were assigned to peptides, with the band at 1070 cm\(^{-1}\) assigned to C–O stretching of carbohydrates (Russell et al., 1983). Intensity ratios of peptide bands to the carbohydrate band were lower in exudates associated with ferricydrite than with goethite, suggesting that carbohydrates were either produced at higher concentrations or were adsorbed to a greater extent than those in the presence of ferricydrite. The IR bands of fungus-derived peptides were not detected in the spectra of mineral-associated BSA.
BSA particles incubated with the fungi (Fig. 2B), perhaps because they were hidden by adsorbed BSA bands and peptides released by hydrolysis. In contrast, carbohydrate bands were observed for the mineral-associated BSA particles, particularly in the case of ferrihydrite.

Fig 2. Modification of ferrihydrite-associated BSA (left) and goethite-associated BSA (right) after incubation with *Piloderma olivaceum* (PIO) and *Hebeloma cylindrosporum* (HEC) for five weeks. Numbers (in mg m\(^{-2}\)) at the left sides of the curves indicate surface BSA concentrations. The ‘0 day’ indicates data from the analysis of the mineral particles before adding the fungi.

A. Size-exclusion chromatograms (area-normalized) of fractions desorbed from iron oxides using a strong phosphate buffer. Shading indicates ±1 SD (n = 2 for 0 day samples and n = 5 for others). Molecular masses (in Da) of peptide standards are indicated at the tops of the panels and are shown as vertical dotted lines.

B. Infrared spectra (mineral intensity-normalized) of the iron oxide-associated BSA. Shaded bands indicate ±1 SD (n = 3). Note that the scales of the y-axes of the ferrihydrite and goethite spectra are different. [Color figure can be viewed at wileyonlinelibrary.com]
Dissolved iron was detected in the culture filtrates collected from the growth media containing iron oxide-associated BSA (Fig. S5). The total dissolved iron was roughly equal to the iron(II) concentration, suggesting that the soluble iron had been released by reductive dissolution. When normalized to the mass of added mineral, more iron(II) was dissolved from ferrihydrite (2.16 mg g\(^{-1}\)) than from goethite (0.25 mg g\(^{-1}\)). From the same mineral, *H. cylindrosporum* tended to dissolve more iron(II) than did *P. olivaceum* (*P* = 0.006 for ferrihydrite and *P* < 0.001 for goethite).

**Proteolysis of mineral-associated BSA**

Culture filtrates with proteolytic activity were collected after growth in medium containing soluble BSA. After five weeks of growth, the culture filtrates showed extensive proteolytic activity (Table S1; Fig. S6A). The levels and specificities of the proteolytic activities of *P. olivaceum* and *H. cylindrosporum* differed slightly, but the activity of both was to a large extent inhibited by the aspartic protease inhibitor pepstatin A (96% of total activity inhibited in *P. olivaceum* and 95% in *H. cylindrosporum*; Table S1). When the collected culture filtrates were incubated with...
mineral-associated BSA, the adsorbed BSA was hydrolyzed in a manner similar to that observed in the in vivo experiments with the fungi (Figs. S7 and S8; cf. Figure 2).

The culture filtrates were fractionated into LMW and high-molecular-weight (HMW) fractions using a membrane with a cutoff of 10 kDa (Fig. S6B and C). Almost all (>98%) proteolytic activity was recovered in the HMW fraction (Table S2). When this fraction was incubated with ferrihydrite- and goethite-associated BSA, proteolysis of the mineral-associated BSA was detected, as shown by a significant reduction in the amount of mineral-associated BSA (Fig. S9). The proteolysis rate increased with increasing volumes of the HMW fractions. The extent of increase was largest with smaller additions and levelled off with larger amendments of the HMW fractions (Fig. S10).

In addition to proteolysis, the reduction of mineral-adsorbed BSA may have been caused by the desorption of BSA via ligand-exchange interactions. To estimate the extent of desorption, iron oxide-associated BSA was incubated with HMW filtrates in which the proteolytic activity was deactivated using pepstatin A (Fig. S11A, B). Upon treatment with the deactivated HMW fraction, less than 1% and 0.5% of the BSA adsorbed on goethite and ferrihydrite respectively, were detected in the aqueous phase surrounding the mineral particles (Fig. S11A). Thus, a substantially lower amount was desorbed by compounds present in the deactivated HMW fraction than was lost by proteolysis of the mineral-associated BSA (cf. Fig. S9).

The effects of the LMW fungal compounds on proteolysis were assessed by incubating the mineral-associated BSA with a given volume of the HMW fraction and increasing volumes of the LMW fractions. Before adding the LMW fraction, the minute proteolytic activity was inhibited by heating. The rate of mineral-associated BSA proteolysis improved with increasing volumes of the LMW fraction regardless of the mineral type, BSA surface concentration, or fungal species (Fig. 4A, Figs. S12A and S13). The enhancement in hydrolysis was increased when the HMW fraction was added to a low, rather than a high, volume of the HMW fraction. This enhancement was not due to ligand-promoted desorption of BSA, as the peak corresponding to BSA was not detected in the SEC chromatogram of the aqueous phase surrounding the mineral particles after incubation with the LMW fractions (Fig. S11C). However, the amounts of certain LMW compounds decreased, suggesting that these had been adsorbed by the mineral particles.

Finally, we examined whether the enhancement of iron oxide-associated BSA proteolysis was due to the effect of species-specific LMW compounds. Notably, LMW fractions from both fungi were equally effective in enhancing HMW fraction proteolysis in P. olivaceum and H. cylindrosporum (Fig. 4 and Fig. S12B).

Discussion

Here, we provide experimental evidence that the ECM fungi H. cylindrosporum and P. olivaceum can access N from iron oxide-associated proteins. Our data suggest that these fungi share a common mechanism with the distantly related species P. involutus for such N access (Wang et al., 2020). We demonstrate that the mechanism involves two components: extracellular aspartic proteases that hydrolyze the protein substrate directly at the mineral surface and exudates that condition the mineral surface to prevent direct adsorption and deactivation of the secreted proteases. Moreover, the enzyme-exudate mechanism applies to proteins associated with both ferrihydrite and goethite, which represent two abundant minerals in forest soils.

Table 1 summarizes our results from H. cylindrosporum and P. olivaceum and compares them with data from P. involutus (Wang et al., 2020). To accommodate the large differences in growth rates between these species, the slow-growing P. olivaceum and H. cylindrosporum were incubated in the medium containing the ferrihydrite- or goethite-associated BSA for a significantly longer time (35 days) than the fast-growing P. involutus (8 days). Previous experiments with P. involutus and Laccaria bicolor showed that assimilation of organic N from SOM depends on the availability of metabolic C (i.e., glucose). The uptake of N ceases when glucose is depleted in the medium, then C-limitation arrests growth and induces autolysis (Nicolás et al., 2019). In the experiments with H. cylindrosporum and P. olivaceum, a substantial fraction, but not the entire amount, of added glucose was assimilated (Table 1). Moreover, no NH₄⁺ was detected in culture filtrates at the end of the incubation period (data not shown), which would have suggested autolysis (Ellström et al., 2015). Collectively, the results indicate that P. olivaceum and H. cylindrosporum were actively growing and did not sense C limitation in the presence of the mineral-associated BSA.

Isotopic analysis of the N content in the mycelia and chemical analysis of the proteins associated with the mineral particles (Figs. 1 and 2) showed that P. olivaceum and H. cylindrosporum hydrolyzed the iron oxide-associated BSA and assimilated the released N. In agreement with data from P. involutus, the assimilation of N from mineral-associated BSA was lower than that from free BSA (Table 1). This can be partly explained by the adsorption of proteases to vacant sites on the mineral surface, leading to the separation of enzyme from its
substrate. This inhibitory effect was counteracted by decreasing the concentration of vacant surface sites, which we accomplished by increasing the surface coverage of BSA (Fig. 1), or by the secretion of mineral surface-reactive compounds (Fig. 4). Additionally, we found that adsorption of proteolytic products on mineral surfaces likely contributed to lower N assimilation from iron oxide-associated BSA than from free BSA. The extent of proteolysis could be increased by increasing the amount of extracellular proteases (Fig. S10) or surface-reactive compounds (Fig. 4). Under the conditions used in our experiments, the assimilation of N from iron oxide-associated BSA by *P. olivaceum* and *H. cylindrosporum* was higher than that by *P. involutus*, particularly at low BSA surface concentrations (Table 1). These differences can be explained by increased production of extracellular proteases and mineral surface-reactive compounds by *P. olivaceum* and *H. cylindrosporum* than by *P. involutus* (Table 1).

In agreement with the data from *P. involutus*, a major part of the proteolytic activity of *P. olivaceum* and *H. cylindrosporum* when grown on soluble BSA was due to secreted aspartate proteases. The collected culture filtrates induced proteolysis of the mineral-associated BSA.

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**Fig 4.** Hydrolysis of ferrihydrite-associated BSA by proteases from *Piloderma olivaceum* (PIO) (left) and *Hebeloma cylindrosporum* (HEC) (right) as a function of added low-molecular-weight (LMW, < 10 kDa) compounds. Proteases were present in high-molecular-weight (HMW, > 10 kDa) fractions of culture filtrates; BSA surface concentration was 0.7 mg m⁻² and the incubation was conducted for 24 h at room temperature. Data are shown as mean ± 1 SD (n = 2). Proteolysis was measured as the decrease in intensity of the BSA peak in size exclusion chromatography, normalized to the intensities of samples incubated with water only.

**A.** Effects of adding increasing volumes of the LMW fraction from the same species that provided the HMW fraction.

**B.** Effects of adding increasing volumes of the LMW fraction from a different species than the one providing the HMW fraction. Data from experiments with goethite-associated BSA at a surface concentration of 0.7 mg m⁻² are shown in Fig. S12. Data from experiments with ferrihydrite- and goethite-associated BSA at a surface concentration of 1.4 mg m⁻² are shown in Fig. S13. [Color figure can be viewed at wileyonlinelibrary.com]
similarly to the fungi. The majority of the proteolytic activity was found in the HMW (> 10 kDa) fraction, which hydrolyzed a substantial part of the mineral-associated BSA. Hydrolysis was abolished when the proteolysis was inhibited by pepstatin A (Fig. S11B). Collectively, the data suggest that aspartate proteases play a key role in the hydrolysis of mineral-associated BSA. The genomes of \textit{P. olivaceum}, \textit{H. cylindrosporum} and \textit{P. involutus} contain a large number of aspartate proteases (Shah et al., 2016). It remains to be determined whether ECM fungi contain aspartate proteases with specific biochemical properties that adapt them to act on substrates adsorbed on mineral surfaces. Moreover, the observation that the majority of the peptides released from the hydrolysis of mineral-associated proteins had molecular sizes over 10 kDa (Fig. S9), suggest that the hydrolysis to assimilable amino acids and oligopeptides also involves the activity of endopeptidases and exopeptidases (Shah et al., 2013).

Similar to \textit{P. involutus}, \textit{P. olivaceum} and \textit{H. cylindrosporum} secreted substantial amounts of mineral-surface reactive compounds during N assimilation from iron oxide-associated BSA. The chemical structures of these compounds are not known in detail, but IR spectroscopy suggested that they are chemically diverse (Table 1). Common to all species were IR bands representing carboxylate moieties. Several ECM fungi, including \textit{P. olivaceum}, \textit{Hebeloma} species and \textit{P. involutus}, can secrete LMW organic acids. These acids, particularly oxalic acid, may cause mineral dissolution and release nutrients via ligand exchange reactions (Lapeyrie et al., 1987; Landeweert et al., 2001; Keiluweit et al., 2015; Schmalenberger et al., 2015; Jilling et al., 2018). From our IR data, we were unable to determine the extent to which oxalic acid contributed to the mineral-surface reactive compounds secreted by \textit{P. olivaceum} and \textit{H. cylindrosporum}. However, compounds promoting ligand-exchange reactions were not secreted in substantial amounts, as BSA was not released from iron oxide upon incubation with protease-deactivated culture filtrates.

The presence of amide I and II bands in the IR spectra and the low C/N ratios of the mineral surface-reactive compounds secreted by \textit{P. olivaceum} and \textit{H. cylindrosporum} indicate the presence of peptides. The nature of these peptides is not clear, but it is well known that ECM fungi, including both used in this study, express a large number of genes encoding secreted peptides including many small secreted proteins (SSPs, < 300 amino acids) (Shah et al., 2016). The C/N ratios of the mineral-surface reactive compounds secreted by \textit{P. involutus} were significantly higher than those from \textit{P. olivaceum} and \textit{H. cylindrosporum}, which may be partly attributed to the secretion of compounds with phenolic functional groups by \textit{P. involutus} (Wang et al., 2017; Wang et al., 2020). Such groups were not detected in the IR spectra of \textit{P. olivaceum} and \textit{H. cylindrosporum}. Difference in the secretion of phenolic compounds between species was expected based on the genes encoding enzymes involved in the biosynthesis of secondary metabolites. The genome of \textit{P. involutus} contains a unique set of genes encoding quinone synthases, which play a key role in the biosynthesis of phenolic pigments (Braesel et al., 2015); however, homologues of these genes are not present in \textit{H. cylindrosporum} and \textit{P. olivaceum} (Shah et al., 2016).

The finding that secreted compounds facilitating the release of N from mineral-associated proteins are not species-specific, i.e., that the mineral surface can be conditioned by exudates produced by another species (Fig. 4), suggests that ECM fungi have evolved mechanisms to keep mineral surface-reactive compounds and enzymes close to their hyphae. During colonisation of mineral surfaces, \textit{P. involutus} hyphae are surrounded by a layer of extracellular substances (Saccone et al., 2012). This layer may have a function similar to that of extracellular polymeric substances (EPS) of bacterial biofilms, which play a major role in mediating adhesion to mineral particles and keep extracellular enzymes and metabolites close to the cell (Flemming and Wingender, 2010). The presence of carbohydrates adsorbed on the mineral, as indicated by IR spectroscopy, suggests that compounds commonly associated with EPS (Flemming and Wingender, 2010) were produced by \textit{H. cylindrosporum} and \textit{P. olivaceum} during N acquisition from iron oxide-associated proteins.

In \textit{P. involutus}, the Fenton reaction facilitates the hydrolysis of soluble BSA. The •OH radical is strongly induced when the fungus switches from ammonium to protein as its primary N source. The •OH oxidizes the protein and extracellular proteolytic activity is initiated shortly thereafter (Op De Beeck et al., 2018). Whether •OH production is induced during the assimilation of N from iron oxide-associated BSA by \textit{P. involutus} is unknown. Such experiments are technically challenging, because they require a method for detecting and quantifying short-lived •OH. Typically, •OH radicals are captured and measured with a probe molecule (Op De Beeck et al., 2018; Yu et al., 2019). We chose not to add such a probe in the experiments with iron oxides, because we have previously reported that they can significantly modify redox reactions at the mineral surface (Lyngsie et al., 2018). However, our data suggest that the capacity to assimilate N from iron oxide-associated proteins is not limited to ECM species that have an extracellular Fenton-based oxidation system. This capacity is also present in species including \textit{H. cylindrosporum} and \textit{P. olivaceum}, which has enzymatic oxidative decomposition mechanisms.
In *P. olivaceum*, *H. cylindrosporum* and *P. involutus* (Wang et al., 2020), the acquisition of N from iron oxide-associated BSA was accompanied by the dissolution of iron oxides. The dissolved iron was mainly present as iron(II), indicating that the dissolution was redox-driven and not promoted by ligand-exchange reactions. In agreement with data from *P. involutus* (Shah et al., 2021), the dissolution of iron oxides by *P. olivaceum* and *H. cylindrosporum* may be accomplished by secreted iron(III)-reducing metabolites. The dissolution was higher in the experiments with *P. involutus* than *P. olivaceum* or *H. cylindrosporum* (Table 1). A higher amount of iron(II) was dissolved from ferrihydrite than from goethite (Fig. S5), which can be attributed to the higher reduction potential of ferrihydrite (Krumina et al., 2017). However, the magnitude of N assimilated from the ferrihydrite- and goethite-associated BSA by *P. olivaceum* and *H. cylindrosporum* was comparable. Further, the release of iron did not increase with the higher uptake of N from iron oxides with large surface coverage. Thus, we could not establish a quantitative relationship between assimilation of N from mineral-associated BSA and reductive iron oxide dissolution. However, the reductive dissolution of iron oxides may play an overall role in nutrient acquisition strategies by promoting the mobilisation of nutrients other than N including mineral associated phosphorus (P) (Shah et al., 2021). Reductive dissolution of iron oxides may also create conditions that can initiate Fenton-like reactions at mineral surfaces and enhancing oxidative decomposition of SOM (Du et al., 2020; Jones et al., 2020).

A key question for future research is how important the enzyme-exudate-driven mechanism is for N nutrition of ECM fungi and subsequently for the host trees (Keiluweit and Kuyper, 2020). Isotope analyses of sporocarps collected in the field (Hobbie et al., 2013; Chen et al., 2016) showed that in certain ECM fungi, the C source for structural biomass was provided by recently assimilated host tree C, whereas an estimated 10% of C in fungal proteins was originated from old soil-derived organic N (Hobbie et al., 2013). Our 13C results corroborate these findings by showing that 4%–11% of mineral-associated BSA C was ended up in fungal mycelium. As mineral-associated organic matter has a significantly longer turnover time than particulate organic matter (Jilling et al., 2018), these data are consistent with our findings that ECM fungi have a molecular toolbox needed for assimilating N from mineral-associated proteinaceous material.

**Experimental procedures**

**Materials**

BSA was chosen as a model protein because of its strong affinity to iron oxide-containing surfaces and its well-known structure, facilitating the interpretation of chemical data (Tian et al., 2020). BSA (purity > 98%, isoelectric point 4.7, MW ~67 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methods for ferrihydrite and goethite synthesis, dialysis and storage as suspensions have been described previously (Krumina et al., 2016). The estimated surface area of ferrihydrite was 300 m² g⁻¹ and the measured surface area of goethite was 62 m² g⁻¹ (Brunauer–Emmett–Teller (BET)) (Krumina et al., 2016). Sterile polyethersulfone membranes (0.22-μm; Millipore, Bedford, MA) were used for filtration and sterilisation of ferrihydrite, BSA solutions and other solutions. Goethite suspensions were sterilized by autoclaving.

**N assimilation from mineral-associated BSA**

*Piloderma olivaceum* (DSMX–4824; PIO) and *Hebeloma cylindrosporum* (Romagnesi TV98IV3; HEC) were maintained on modified Fries medium (SI, S3.1) containing 1% agar. Nitrogen assimilation from mineral-associated BSA was assayed as detailed by Wang et al. (2020). Briefly, a mycelial plug of ~4 mm was inoculated in Petri dishes on a layer of glass beads immersed in 10 ml modified Fries medium. Fungi were incubated for 5 weeks at 18°C in the dark. The medium was then replaced with Fries medium without N to induce N-deprived mycelia for 24 h (Shah et al., 2013). Mycelia were washed with Milli-Q (MQ) water and the fungi were incubated with 10 ml of media containing soluble (i.e., free) BSA, mineral-associated BSA, or mineral without associated BSA, for 5 weeks. Mycelia were labelled with 13C and 15N by adding 13C-o-glucose (~5.6 atom%) and 15N-ammonium (~2.3 atom% 15N) to both the Fries and the BSA medium.

Media containing iron oxide-associated BSA were prepared at pH 3.5, which is typical of boreal forest soils. The suspensions of ferrihydrite or goethite were mixed with a BSA stock solution. The final solid concentrations of ferrihydrite and goethite were 0.5 and 1 g L⁻¹ respectively. All media contained 0.01 M KCl, 3 g L⁻¹ 13C-o-glucose, 10 mg l⁻¹ 15N-ammonium-N (five-fold less than in the Fries medium) and vitamins in concentrations similar to those in a Fries medium. Ammonium was added because the production of extracellular proteases in *P. involutus* is strongly induced by the switch from ammonium to BSA as the N source (Op De Beeck et al., 2018). All media were equilibrated for 24 h to enable BSA adsorption under continuous stirring. For each mineral type, two BSA surface concentrations were prepared: 0.7 and 1.4 mg BSA m⁻². At these concentrations, all BSA was adsorbed and no desorption of BSA was detected (Tian et al., 2020; Wang et al., 2020). We also prepared media containing ferrihydrite or goethite without BSA, as
Decomposition of mineral-associated proteins

well as mineral-free medium with soluble BSA at a concentration of 100 mg L⁻¹. This concentration corresponds to the amount of BSA added in the goethite-associated BSA medium at a surface concentration of 1.4 mg BSA m⁻² and ferricydrate-associated BSA medium at 0.7 mg BSA m⁻². 

After 5 weeks of incubation (18°C in the dark), mycelia were collected, washed thoroughly with MQ water and lyophilized for the measurement of fungal biomass, elemental (C and N) and isotopic compositions (¹³C and ¹⁵N). The media containing minerals were centrifuged to separate the liquid (i.e., culture filtrates) and mineral phases.

Secreted compounds and mineral dissolution

Mineral phases recovered from the mineral-associated BSA media were lyophilized and analysed for total C, N, atom% ¹³C and atom% ¹⁵N. From these values, the amounts of C and N secreted by the fungi and adsorbed on the minerals were estimated using isotope-mixing models (Wang et al., 2020). The secretion of mineralsurface reactive compounds when H. cylindrosporum or P. olivaceum was grown on free BSA was determined by incubating an aliquot of culture filtrate with a suspension of goethite for 24 h (SI, S3.2). Iron mineral dissolution was examined by measuring dissolved total Fe and Fe²⁺ in culture filtrates. Compositions of organic compounds adsorbed on the mineral surfaces were analysed using IR spectroscopy (VERTEX 80v, Bruker, Karlsruhe, Germany).

Proteolysis of mineral-associated BSA in vitro

Culture filtrates were collected after growing H. cylindrosporum and P. olivaceum for 5 weeks on medium containing soluble BSA. Their proteolytic activities at pH 3.5 were analysed using BSA or fluorescence-labelled casein as substrates (Wang et al., 2020). The capacity of the culture filtrates to hydrolyze the mineral-associated BSA at surface concentrations of 0.7 1.4 mg BSA m⁻² was tested by adding 0.8 or 1.6 ml of filtrates into 2 ml Eppendorf micro-centrifuge tubes containing either 0.2 mg ferricydrate or 0.4 mg goethite that was associated with BSA. The total volume was adjusted to 1.6 ml with MQ water. The suspensions were incubated for 24 h at 20°C in the dark with continuous end-over-end shaking. For comparison, incubations were also conducted in the presence the protease inhibitor pepstatin A (Sigma-Aldrich, final concentration 10 mg L⁻¹). 

Culture filtrates were fractionated into HMW (> 10 kDa) and an LMW (< 10 kDa) fractions using centrifugal concentrators with a cutoff of 10 kDa (Filtron Tech., Northborough, MA). Increasing volumes of the HMW fraction were incubated with iron oxide-associated BSA. Incubations were also conducted with HMW fractions (100 μl of H. cylindrosporum and 50 μl of P. olivaceum) treated with pepstatin A. Total volumes were adjusted to 1.2 ml by adding MQ water and incubated for 24 h as described above.

Effects of the HMW fractions on proteolysis were examined by adding increasing volumes of heat-deactivated (65°C for 45 min) HMW fraction into micro-centrifuge tubes containing mineral-associated BSA. Volumes were adjusted to 1.2 ml with MQ water and the suspensions were equilibrated for 30 min. Subsequently, a range of volumes (0, 20 and 50 μl for both fungi; 10 for H. cylindrosporum and 140 for P. olivaceum) of the HMW fraction were added. The samples were incubated for 24 h as described above. Similar experiments were conducted by combining HMW and HMW fractions from different species. A heat-deactivated HMW fraction (0, 0.3, or 0.6 ml) from H. cylindrosporum was incubated with 20 μl of the HMW fraction from P. olivaceum; further, 0.6 ml of the heat-deactivated HMW fraction from P. olivaceum was incubated with 10 μl of the HMW fraction from H. cylindrosporum.

After incubation with the culture filtrates, the mineral and liquid phases were separated by centrifugation. Aliquots of the mineral particles from the incubations with the non-fractionated culture filtrates were analysed by IR. Mineral particles were incubated in 1 ml phosphate buffer (0.5 M phosphate and 1.5 M NaCl, pH 7.2) for 30 min to desorb compounds from the mineral surfaces (Wang et al., 2020) and the liquid phases generated were analysed by SEC.

Chemical analyses, data analysis and statistics

The elemental compositions (total C, N, ¹³C atom% and ¹⁵N atom %) of fungal mycelia and mineral particles were analysed by elemental analyzer-isotope ratio mass spectrometry. Mineral particles were characterized by attenuated total reflectance (ATR) IR spectroscopy (Wang et al., 2019). Dissolved Fe and Fe²⁺ in the culture filtrates were measured by the ferrozine method. Size distributions of compounds in culture filtrates and other liquid phases were analysed using SEC at 214 nm. Isotopic calculations were performed based on previously published equations (Wang et al., 2020). IR spectra of goethite particles were normalized using the goethite bands (peaks at 790 and 892 cm⁻¹) after the min-max normalisation function; those of ferricydrate were normalized using the broad ferricydrate bands in the region 877–531 cm⁻¹ after a vector normalisation function using OPUS software (v 7.2, Bruker). Chromatograms were normalized to the areas under the curves ranging from

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8 to 18 min. Further details on chemical analyses are given in the Supplemental Information (S3.3).

Two-way analysis of variance was conducted using SPSS software (v18; SPSS, Chicago, IL) to test the effects of fungal species and BSA surface concentrations on biomass properties (C, N and 15N), assimilated N fraction, mineral-associated C and N that were produced by the fungi, and dissolved Fe.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Appendix S1**: Supplementary Information