Letter

Interaction between tannins and fungal necromass stabilizes fungal residues in boreal forest soils

Introduction

Boreal forest ecosystems store significant amounts of the global carbon (C) pool, with a major fraction stored belowground. Mechanisms controlling the stability of C in belowground organic matter are not well identified, hindering the understanding of the global C cycle and how it affects the future climate. Emerging views accentuate the role of microorganisms in organic matter accumulation. In this work we combine laboratory and field experiments, and propose a mechanistic explanation for the stabilization of fungal necromass based on its interaction with tannins, a group of plant secondary metabolites well-known for their ability to precipitate proteins and potential role in ecosystem functioning. In our laboratory study with pine (*Pinus sylvestris*) microcosms decomposition of fungal necromass was decreased by the formation of complexes with tannins. Our field study demonstrated that fungal necromass–tannin complexes may be created also under natural conditions. Our results highlight that interaction between tannins and fungal necromass as a hitherto overlooked mechanism that stabilizes microbial-derived C in boreal forest soils.

The need of understanding the mechanisms behind C stabilization into slowly cycling pools has become urgent due to the threat of global climate change (Hopkins *et al.*, 2012, 2014; Liang *et al.*, 2017). Taken globally, almost one-third of the forest C stock is stored in boreal ecosystems (Pan *et al.*, 2011), whereof 60% has accumulated in the soil, mainly in soil organic matter (Lehmann & Kleber, 2015). However, the fate of this C under climate change is uncertain as soils may shift from being C sinks to C sources, thereby accelerating global warming (Crowther *et al.*, 2016).

Ecologists over the past decades have proposed numerous hypotheses aiming at deciphering the main sources of persistent soil C and the mechanisms behind its stabilization. Although input of aboveground plant litter for a long time was considered the main origin of persistent organic matter, more recent studies point to plant belowground input and fungal necromass as significant contributors of the stable organic matter pool in forest soils (Baldrian *et al.*, 2013; Clemmensen *et al.*, 2013; Ekblad *et al.*, 2013; Kyaschenko *et al.*, 2017a, 2018; Sun *et al.*, 2018). Persistence of these C inputs may be explained by chemical, environmental and biological factors (Schmidt *et al.*, 2011). It has been hypothesized that chemically recalcitrant C inputs, i.e. for the plant litter mainly lignin and lipids (Melillo *et al.*, 1982) and, for the fungal chemistry, the abundant polysaccharide chitin, and polymers of phenolic and indolic monomers – melanin form the most stable organic matter (Fernandez & Koide, 2012; Clemmensen *et al.*, 2015; Fernandez & Kennedy, 2018). However, some studies have shown that lignin turnover in the soil is more rapid than the bulk of the organic matter, and fungal necromass and chitin degradability is relatively high (Godbold *et al.*, 2006; Drigo *et al.*, 2012; Fernandez & Koide, 2012, 2014; Brabcová *et al.*, 2018). Moreover, it has been proposed that both recalcitrant and labile C inputs build up persistent organic matter and these C inputs can be stabilized by interactions with soil minerals (Mikutta *et al.*, 2006; Cotrufo *et al.*, 2015). A concept of a microbial C pump proposes that microbial turnover results in deposition of microbial-derived C (Liang *et al.*, 2017). Recent studies in boreal forests highlight enzymatic oxidation and fungal communities as important regulators of organic matter accumulation in the mor layer (Clemmensen *et al.*, 2015; Kyaschenko *et al.*, 2017b; Stendahl *et al.*, 2017).

The earlier-mentioned framework of C stabilization mechanisms should also incorporate possible biochemical interactions between microbial and plant C inputs. For example, condensed tannins are an abundant group of plant polyphenols, which can affect organic matter decomposition (Northup *et al.*, 1995; Kraus *et al.*, 2003; Adamczyk *et al.*, 2014; Sun *et al.*, 2018) and nutrient cycling (Hättenschwiler & Vitousek, 2000) through the formation of complexes with proteins (Bending & Read, 1996; Hagerman, 2012) and possibly also with chitin (Adamczyk *et al.*, 2011, 2013). These complexes of chitin and proteins from fungal biomass with tannins of plant origin could have a major role in the stabilization of soil C and the accumulation of organic matter. This pathway of soil C stabilization could be particularly important in boreal ecosystems with their typically tannin-rich plant roots (e.g. Adamczyk *et al.*, 2016) associated with abundant ectomycorrhizae that can reach a fungal biomass of up to 600 kg ha^{-1} (Wallander *et al.*, 2004). With reference to these factors, we tested whether fungal necromass–tannin complex formation could be a yet overlooked mechanism of organic matter stabilization. In a laboratory experiment, we compared decomposition of fungal necromass and fungal necromass–tannin complexes and the subsequent uptake of nitrogen-15 (^{15}N) by mycorrhizal pine (*Pinus sylvestris*) seedlings. We hypothesized that decomposition and uptake of N from fungal necromass–tannin complexes would be slower in comparison to uncomplexed fungal necromass (Hypothesis 1). The field experiment was conducted using fungal necromass-containing mesh bags. Within the field experiment, we hypothesized that condensed tannins from roots would transform fungal residues into more persistent forms slowing down fungal necromass decomposition (Hypothesis 2),

and that fungal necromass–tannin complexes would be created also under natural conditions (Hypothesis 3).

Materials and Methods

Pine (*Pinus sylvestris* L.) seedlings were placed in Perspex® microcosms filled with sieved material from the soil organic layer collected from the SMEAR II station, Finland (61°84′N, 24°26′E) (Hari *et al.*, 2013). The microcosms were placed in growth chambers. Wet fungal necromass–tannin complexes in mesh bags (50 µm mesh size, 1 cm × 1 cm) were transferred to the microcosms. After the experiment, the soil was thoroughly colonized by roots and root-associated fungi (Supporting Information Fig. S1). We used the following treatments: (1) mesh bags with fungal necromass, (2) mesh bags with fungal necromass together with bound tannins. We have used five microcosms per treatment. The fungal biomass (basidiomycete, *Dichomitus squalens*), used to obtain fungal necromass, was cultivated on Hagem's liquid medium with ^{15}N-labelled ammonium chloride (NH_4Cl. For the field experiment, the fungal biomass was not labelled. Initial chitin (Ekblad & Näsholm, 1996) and melanin (Fernandez & Koide, 2014) content of fungal necromass was measured. Complexes between the fungal necromass and condensed tannins were generated by mixing fungal necromass with condensed tannins (for tannin purification, characterization and complex preparation see Supporting Information, Table S1; Figs S2–S5). Soil samples, mesh bags and plant material were collected from the microcosms after 3.5 months of incubation. We measured protease, laccase and chitinase activities (Shah *et al.*, 2013; Adamczyk *et al.*, 2016) and chitin concentration (Ekblad & Näsholm, 1996; Fig. S6) inside the mesh bags. The ^{15}N content of seedlings and soil was analysed by isotope-ratio mass spectrometry coupled to an elemental analyser (Thermo Finnigan, Hampton, NH, USA).

In the field experiment, we placed fungal necromass (3.0 g dry weight) into mesh bags and buried them between the organic and topmost mineral soil horizons relative to the horizon position at three different sampling sites (within-site replication) at the SMEAR II station, Finland. We used mesh bags of different mesh sizes: 1 mm (not limiting root and hyphal in-growth), 50 µm (excluding roots but not fungal hyphae) and 1 µm (excluding also fungal penetration). The mesh bags were collected in September after the first, second and third growing seasons. We measured mass loss, total amount of tannins inside the mesh bags and the amount of bound tannins (Hagerman & Butler, 1978; Hagerman, 2012). Detailed description of the materials and methods and statistical analysis are given in Supporting Information (Methods S1, S2).

Results and Discussion

In microcosms, the total mass loss and chitin loss from fungal necromass were higher for the untreated fungal necromass than for the fungal necromass–tannin complexes ($P < 0.001$) (Fig. 1), which is in line with Hypothesis 1 that slower decomposition of fungal necromass would occur in the presence of tannins. One may assume that lower decomposition rate is a result of the

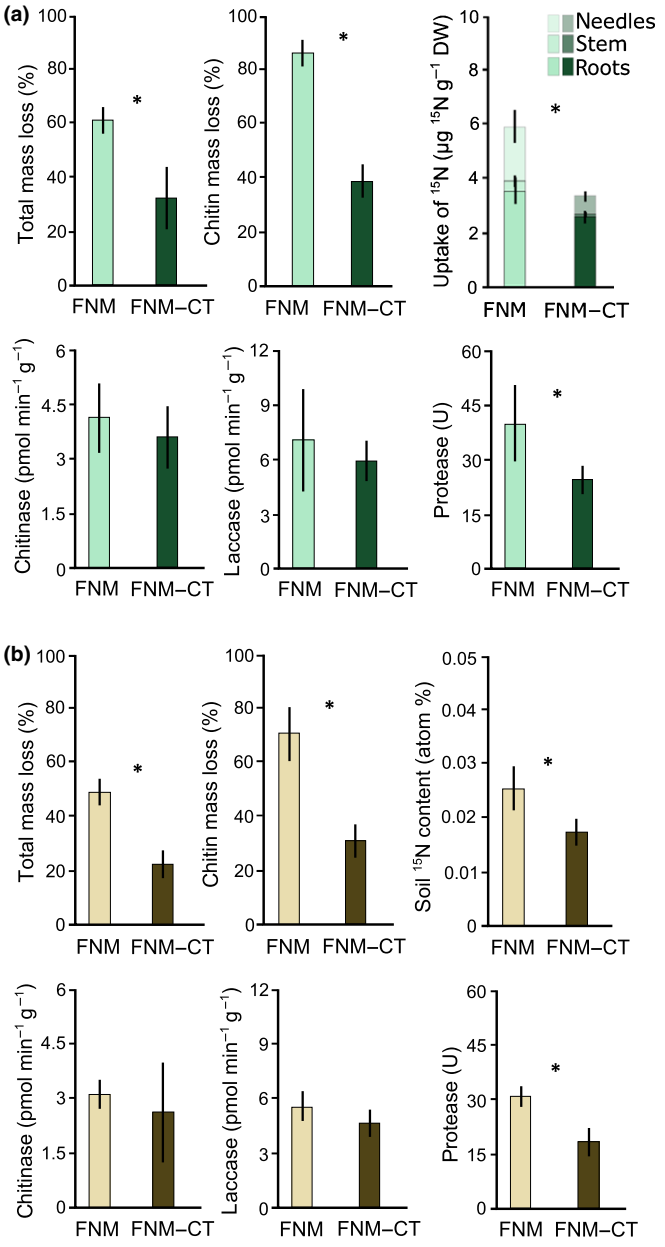

Fig. 1 (a) Total mass loss and chitin loss of untreated fungal necromass compared with fungal necromass–tannin complexes, uptake of nitrogen-15 (^{15}N) from labelled fungal necromass by seedlings, and enzymatic activities in mesh bags containing fungal necromass in laboratory microcosm with pine (*Pinus sylvestris*) seedlings growing in organic boreal forest soil. (b) Total mass loss and chitin loss of fungal necromass compared with fungal necromass–tannin complexes, soil content of ^{15}N originating from introduced labelled fungal necromass and enzymatic activities in mesh bags in nonplanted laboratory microcosms. At the beginning of the experiment the amount of fungal necromass in the mesh bags was 10 mg (dry weight, DW) and the amount of condensed tannins in the fungal necromass–tannin complexes was 0.45 mg. The chitin and melanin concentration of fungal necromass at the beginning of the experiment was 12.5% and 7%, respectively. The error bars indicate ± SE of the mean. To determine significant differences between treatments (fungal necromass and fungal necromass–tannin complexes) in mass loss, chitin loss, ^{15}N uptake and enzymatic activities *t*-tests were used. Significant differences ($P < 0.05$) between treatments are indicated by asterisks. FNM, fungal necromass; FNM–CT, fungal necromass–condensed tannin complexes.

inhibitory effect of tannins on activities of decomposer enzymes (Triebwasser *et al.*, 2012; Adamczyk *et al.*, 2017), but we observed only a slight decrease in activities of some measured extracellular enzymes in response to the tannin treatment (Fig. 1a). Moreover, condensed tannins were tightly bound to the fungal necromass (Figs S2–S4), leaving little opportunity for interaction with enzymes. These circumstances point to that slower fungal necromass decomposition emerges rather from the formation of complexes with condensed tannins, and not from inhibition of enzymes. Uptake of ^{15}N from labelled mycelium into the seedlings was always higher from untreated fungal necromass than from the fungal necromass–tannin complexes ($P < 0.05$) (Fig. 1a), which underlines that tannins transform fungal necromass into a less available source of N. The soil was also more ^{15}N enriched in nonplanted microcosms with untreated fungal necromass than in those with fungal necromass–tannin complexes ($P < 0.05$) (Fig. 1b). Although the results from our microcosm study proposed that condensed tannins may effectively slow down fungal necromass decomposition, this mechanism remained to be confirmed at the field scale.

We assumed that formation of fungal necromass–tannin complexes may be of particular importance in boreal forests due to the following reasons: (1) boreal forest plants and soils are tannin-rich (Smolander *et al.*, 2012) and the purely organic mor layer is rich in fungal biomass (Wallander *et al.*, 2004), (2) there are high amounts of tannin-reactive N compounds, that is protein and chitin, in the fungal biomass (Adamczyk *et al.*, 2011; Zeglin *et al.*, 2013), (3) soil pH is low, and organic N compounds primarily interact with tannins at pH levels below six (Adamczyk *et al.*, 2011), typical for the boreal forest soil (Högberg *et al.*, 2007; Calvo-Polanco *et al.*, 2017). Accordingly, we sought for direct experimental evidence for the creation of fungal necromass–tannin complexes at the field scale. We placed fungal necromass into mesh bags with different mesh sizes to disentangle the effects of bacteria, fungi, and roots, and buried them in the soil organic layer at the SMEAR II station, Finland. The rate of fungal necromass decomposition during the first year, measured as loss of total mass of mycelium was high (above 85%) in all of the treatments (Fig. 2a). Fast rates have been observed previously during the initial phases of fungal necromass decomposition (e.g. Brabcová *et al.*, 2016), in line with that, the C : N ratio of the fungal necromass used in our study was far lower than that of the soil (13 vs 30), presumably making the fungal necromass an attractive N source in generally N-limited boreal soil (Vitousek & Howarth, 1991; Magnani *et al.*, 2007). After the first year we did not observe differences between decomposition in 1 µm bags and 50 µm or 1 mm bags. Although the 1 µm mesh size effectively excluded fungal ingrowth, as indicated by negligible concentrations of ergosterol (marker of living fungal biomass), the long incubation time might have concealed potential differences between bacterial decomposers (1 µm) and fungal decomposers (50 µm and 1 mm). After the second and third year mass loss continued to progress in the 50 µm and 1 µm mesh bags ($F = 45.90$, $P < 0.001$; $F = 50.31$, $P < 0.001$, respectively) (Fig. 2a), but in the 1 mm mesh bags with fine root access, no further significant mass loss occurred ($F = 0.70$, $P = 0.40$). This result suggests that tannins from fine root litter

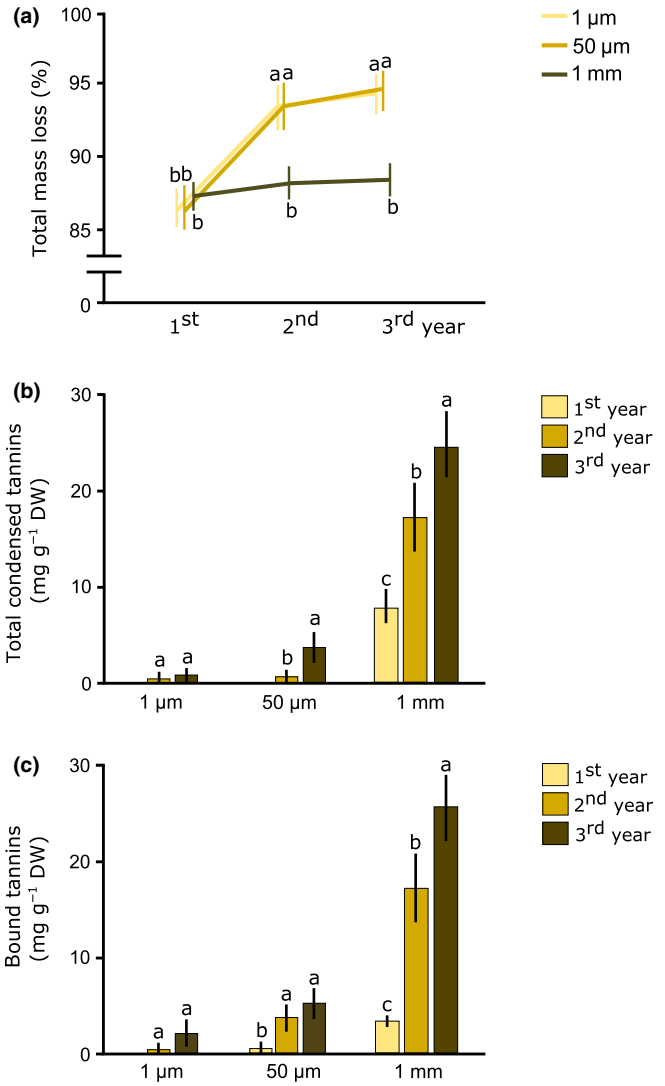

Fig. 2 (a) Loss of total dry mass of fungal necromass (originally 3 g dry weight, DW), (b) total amount of condensed tannins, and (c) amount of fungal necromass–bound tannins in mesh bags incubated for up to three years in the soil of a boreal forest in a *Pinus sylvestris* stand. The error bars indicate ± SE of the mean. To determine significant differences between the treatments, we constructed linear mixed-effects models with mesh size and incubation time as fixed factors and site as a random factor. As a *post hoc* test we used Tukey test. Site effects were always nonsignificant. Significant differences ($P < 0.05$) between treatments within the same year are indicated by different letters.

formed complexes with fungal necromass, slowing down its decomposition, which would agree with Hypothesis 2. It seems plausible that formation of fungal necromass–tannin complexes and the subsequent decrease of decomposition rate may have started even earlier than after one year. Under natural conditions, decomposition may be hampered also in more freshly formed mycelial necromass, where mycorrhizal fungi are in closer connection with tannin-rich roots. As we used 3 g of fungal necromass, underestimation of mass loss due to ingrowth of fine roots (1 mm) and mycorrhizal mycelium (1 mm and 50 µm) should be of rather small importance. The amount of condensed tannins in the mesh bags was increasing with time of incubation, especially in the 1 mm

treatment ($F = 32.71$, $P < 0.001$), supporting the possibility of fungal necromass–tannin complex formation in the presence of fine roots (Fig. 2b). Also, in the 50 µm and 1 µm treatments tannin concentrations increased, as the mesh bags did not exclude entrance of condensed tannins from the surrounding soil, but these values were significantly lower than in the 1 mm mesh bags, especially after three years of incubation ($P < 0.001$). Taking into account previous reports that fine roots contributed two-thirds of the condensed tannins in the soil (Xia *et al.*, 2015) and that fine roots were found in all of the 1 mm mesh bags, we conclude that root ingrowth was the main source of condensed tannins, not the surrounding soil. To directly confirm that tannins in the mesh bags formed complexes with the fungal necromass we applied a protein-precipitation method, which is used to estimate protein-bound tannins (Hagerman, 2012). After removal of potentially unbound tannins with water and water–methanol, we added triethanolamine (TEA), which releases bound tannins (Fig. S5). The results confirmed that the tannins found in the 1 mm bags were bound to the fungal necromass (Fig. 2c), providing a direct evidence for fungal necromass–tannin complex formation under field conditions and supporting Hypothesis 3. We recognize that other processes than fungal necromass–tannin complex formation could partially have affected mass loss in the 1 mm mesh bags. For example, competition between roots and decomposers for limiting N may have resulted in reduction of decomposer activity, but taking the large amounts of fungal necromass (3 g) into account, this effect should be of rather minor relevance. In addition, uptake of water by roots in the 1 mm bags may have affected decomposition, but moisture content in mesh bags after incubation in soil was at similar levels in all treatments. It is also possible that the tannins in the mesh bags operated partly via a different route, as tannins may act as microbial inhibitors or stimulators (Schimel *et al.*, 1996; Fierer *et al.*, 2001). However, it was shown that low molecular mass tannin fractions act as microbial stimulators, and high molecular mass tannins as inhibitors, but primarily by binding extracellular substrates (Fierer *et al.*, 2001; Kanerva *et al.*, 2006), which supports our results. Moreover, according to our results, the mesh bags contained only fungal necromass-bound tannins, reflecting our microcosm study, in which interactions with microbial enzymes were limited.

Conclusions

Overall, our results supported the idea that complex formation with root-derived tannins may contribute to stabilization of fungal necromass in boreal forest soils. Our experiments provide a potential mechanistic explanation to previously reported stabilization of C originating from fungal mycelium and root litter (Clemmensen *et al.*, 2013), the very slow decomposition of mycorrhizal first-order roots (Sun *et al.*, 2018) and N retention driven by root-derived C (Kyaschenko *et al.*, 2018). Decreased decomposition of fungal necromass by interaction with tannins also fits well into the recently proposed concept of the microbial C pump, where microbial turnover results in deposition of microbial-derived C (Liang *et al.*, 2017). Future studies ought to consider differences in biochemistry between fungal species, for example, their content of proteins and polysaccharides, such as chitin, which may interact with tannins. Also studies of tannin–fungal necromass stability over long periods (years to millennia) should be taken into account. In addition, as the fungal necromass decay rate depends on the microbial communities associated with the decomposing mycelium (Brabcová *et al.*, 2016; Fernandez & Kennedy, 2018), further research should also involve in-depth studies of decomposer community structures and their capabilities to attack and brake up tannin complexes.

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Author contributions

BA and JH planned and designed the research, BA, O-MS, and CB performed experiments and data analysis. BA wrote the first draft of the manuscript and all authors contributed to revisions.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

Fig. S1 Pine seedling in microcosm with abundant ectomycorrhizal hyphae.

Fig. S2 Formation of fungal necromass–tannin complexes.

Fig. S3 Formation of fungal necromass–tannin complexes; the amount of tannins in precipitates.

Fig. S4 Effect of chitin and fungal necromass on acid–butanol assay.

Fig. S5 Stability of fungal necromass–condensed tannin complexes.

Fig. S6 Chromatogram of chitin (its monomer, glucosamine) from fungal necromass.

Methods S1 Precise description of experimental design of laboratory and field experiments.

Methods S2 Formation and stability of fungal necromass–tannin complexes.

Table S1 Characterization of condensed tannins used in the study.

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Key words: boreal forest, fungal necromass, nitrogen uptake, organic matter accumulation, plant–soil interaction.