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Ectomycorrhizal fungi in association with *Pinus sylvestris* seedlings promote soil aggregation and soil water repellency

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ABSTRACT

Research on fungal effects on soil aggregation has been heavily biased towards arbuscular mycorrhiza. Even though ectomycorrhizal fungi are thought to be as important as arbuscular mycorrhizal fungi and saprotrophic fungi in contributing to soil structure, there are few experimental studies on this topic. Here we quantified how nine ectomycorrhizal fungi in association with *Pinus sylvestris* seedlings affected soil aggregation and soil water repellency (SWR) of a sandy loamy soil. Water-stable aggregates (>0.25 mm diameter) increased by 6–12% when plants were associated with *Laccaria bicolor*, *Laccaria laccata*, *Lactarius theiogalus*, *Paxillus involutus* and *Suillus bovinus*. Mean weight diameter (MWD) of soil aggregates also increased, primarily in the 2–4 mm diameter size class. However, *Suillus granulatus* increased water-stable aggregates but not MWD, conversely *Rhizopogon roseolus* and *Suillus luteus* increased MWD but not water-stable aggregates. We also found *Lt. theiogalus*, *R. roseolus* and *S. luteus* promoted SWR. Furthermore, hyphal length was weakly correlated with MWD ($R = 0.27$, $P < 0.05$), especially with aggregate mass in the 2–4 mm size class ($R = 0.32$, $P < 0.05$). However, we could not identify clear soil effects (soil pH, soil protein content) serving as explanation for either soil aggregation or SWR. Thus, we conclude that interactions between fungi and soil structure are a species-dependent processes based on yet to be characterized fungal traits. Our results have added further evidence from direct experimentation that ectomycorrhizal fungi can contribute to soil aggregation and SWR.

1. Introduction

As important terrestrial mutualistic fungal groups, ectomycorrhizal (EcM) fungi and arbuscular mycorrhizal (AM) fungi have been intensively studied regarding their effects on plant growth, plant communities and ecosystem processes (Smith and Read, 2008). Nevertheless, concerning the relationship between soil fungi and soil aggregation, research has been predominantly focused on AM fungi (Tisdall and Oades, 1982; Rillig and Mummey, 2006), perhaps because of the widely accepted importance of this process in agroecosystems and grasslands, in which AM fungi predominate.

Despite this imbalance in research, several lines of evidence suggest that EcM fungi may be as important as AM fungi in improving soil structure. Firstly, filamentous soil fungi, including EcM fungi, saprophytic fungi and others should simply be able to influence soil structure by virtue of their hyphal growth habit, which could enmesh particles and bind them into soil aggregates (Tisdall and Oades, 1982; Tisdall et al., 1997; Ritz and Young, 2004; Six et al., 2004; Rillig and Mummey, 2006). Secondly, already Thornton et al. (1956) observed that mycelia aggregated sandy soil under *Pinus radiata*, and more recently Caesar-Tonthat et al. (2013) observed that soil aggregation increased in the zone adjacent to *Agaricus lilaceps* (an EcM fungus) fairy rings.

Thirdly, some limited results show that EcM fungi and saprotrophic fungi and their extracellular exudates promoted soil water-stable aggregates (WSA). For example, *Pisolithus tinctorius* (an EcM fungus) when colonizing *Fraxinus uhdei* increased WSA of the 0.5–1 mm diameter fraction of a sandy clay loam by 3% (Ambriz et al., 2010). Saprotrophically growing EcM fungi also promoted soil aggregation to different degrees (Graf and Gerber, 1997; Graf et al., 2006), and in an experiment using clay particles smaller than 2 μm, the EcM fungus *Hebeloma* sp. and the saprotrophic fungus *Rhizoctonia solani* both increased aggregation >50 μm

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significantly, with *Hebeloma* sp. having a smaller effect (Tisdall et al., 1997). In a non-sterile soil, unidentified saprotrophic fungi had a significant, positive effect on macroaggregate formation (Denef et al., 2001). Exuded extracellular mucilage from saprotrophic basidiomycete fungi, likely polysaccharides, has been related to soil aggregate water stability (Caesar-Tonthat, 2002). Mucilage from Trichocomaceae (Ascomycota) showed similar results and 6 isolates of this group of fungi increased the mean weight diameter (MWD) of soil aggregates of inoculated soil by more than 12%, although the effect was transient and decreased after 3 weeks (Daynes et al., 2012). Thus, even though direct evidence for soil aggregation by symbiotically growing EcM fungi is very rare, it is highly likely that soil aggregate formation by fungal mycelia is a process mediated by many types of filamentous fungi, including EcM fungi.

Among fungal exudates, hydrophobic compounds may be especially important for soil aggregation. Some EcM fungal hyphae are hydrophobic, which presumably helps fungi transport nutrients and water while exploring larger distances in soil (Unestam and Sun, 1995; Agerer, 2001). They could produce hydrophobins, which are small hydrophobic proteins, having multiple functions in mycelium growth, fruiting body formation, and the alteration of surface polarity (Wessels, 2000; Wösten and Vocht, 2000; Linder et al., 2005). These hydrophobic compounds are thought to have an additional function of affecting soil wettability and inducing soil water repellency (SWR) (Rillig, 2005; Hallett, 2007; Diehl, 2013). For example, in forests SWR under *Pinus* was closely related to fungal activities (Lozano et al., 2013).

There has been a rapid increase in research on SWR (Dekker et al., 2005), not only focused on the role of fungi, but also concerning the relationship between SWR and water stable soil aggregation (Bisdom et al., 1993; Vogelmann et al., 2013). The proposed mechanism for this relationship is that increasingly hydrophobic soil organic matter (SOM) could prevent breakage of dry soil aggregates during rewetting, therefore creating more water-stable aggregates (Piccolo and Mbagwu, 1999; Six et al., 2004). It is not yet clear whether or not the fungal associated SOM contributing to SWR and the SOM contributing to soil aggregation are the same, but Rillig (2005) has proposed that hydrophobins may be involved in both processes. Thus, it is important to conceptualize soil aggregation and SWR as two processes simultaneously influenced by EcM fungi.

In this research, soil aggregation and SWR were studied as functions of EcM in order to understand relationships among EcM fungal mycelia, soil structure, and soil moisture. The objectives of this study were: (1) to test whether nine commonly studied EcM fungi in association with *Pinus sylvestris* seedlings were able to promote soil aggregation and SWR, (2) to test whether pH (a key factor in SWR; Diehl, 2013) or soil protein content were related to soil aggregation and SWR, and (3) to test whether SWR was related to soil aggregation.

2. Materials and methods

2.1. Soil

Soil was collected from a meadow near an experimental field of Freie Universität Berlin. Soil at the site was an Albic Luvisol with the following properties: sand = 74%, silt = 18% and clay = 8%; 64% initial WSA; pH($CaCl_2$) = 7; 6.9 mg/100 g P (calcium–acetate–lactate); 5.0 mg/100 g K (calcium–acetate–lactate); 0.12% N (total); 1.87% C (total) (analyses conducted by LUFA Rostock Agricultural Analysis and Research Institute, Germany; and using a Euro EA C/N analyzer, HEKAtech GmbH, Wegberg, Germany) (Rillig et al., 2010). Soil was sieved (10 mm) to remove stones and roots. Following that, the soil was steamed at 80 °C (8 h) to eliminate fungi and soil animals. Soil was then air dried and sieved to pass a 4 mm sieve to remove smaller roots and twigs and to further homogenize the soil.

2.2. Fungi and seedlings cultivation

Nine ectomycorrhizal fungal isolates from five families (Table 1) were chosen to test the effects of EcM fungi on soil aggregation. Six of them were kindly provided by Prof. A. Polle (Georg-August University Göttingen), the others were obtained from the Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre (http://www.cbs.knaw.nl/). These EcM fungi were selected because of their availability and record of intensive study. Scots pine, *P. sylvestris*, was chosen as the host plant because it can be colonized by all our EcM fungi (Colpaert et al., 1992, 1996). Commercial seeds were from Forstsaatgut-Beratungsstelle (Münster, Germany).

Modified Melin-Norkrans' (MMN) medium (Kottke et al., 1987) was used to cultivate EcM fungi. In order to prevent mycelia from growing inside the agar a sheet of cellophane was autoclaved in water at 121 °C for 5 min, and then placed on the surface of agar (Cassago et al., 2002). Afterwards two mycelium plugs were placed on the cellophane and grown at 25 °C for 3 weeks to produce inocula. In the control, only a sheet of sterilized cellophane was added without fungal plugs. Scots pine seeds were surface sterilized in 30% H_2O_2 for 15min, rinsed thoroughly, soaked in water for 4 h, and then added to Petri dishes with 1% water agar sealed with parafilm to maintain moisture and sterility during germination. Ten days later 200 germinated seeds were sown into 10 boxes (16 cm × 11 cm × 6 cm, L × W × H) with a sterilized perlite and vermiculite mixture (1:1); each box had 20 seedlings. Seedlings were watered by tap water twice a week. After 4 weeks pre-growth in the climate chamber (75% humidity; 24/16 °C, day/night temperature), the seedlings were inoculated.

2.3. Inoculation and growth

Roots of seedlings were inoculated with EcM fungi using a "sandwich" method (Colpaert et al., 1996). Briefly, roots of a seedling were spread on a piece of cellophane with mycelia (treatment) or without (control), keeping roots in contact with inocula in case of treatment, covering them with two pieces of filter paper (Rotilabo-Rundfilter, Typ111A) soaked in modified MMN liquid medium, and then inoculated for 5 days. Seedlings were randomly assigned to treatments, at which point seedling fresh weight was recorded (before inoculation) for subsequent use of this measure as a covariate. Then each seedling was transplanted into a bleached plastic pot (1 L, 13 cm × 14 cm, W × H) with 800 g prepared soil. In all, there were nine fungal treatments and one control, replicated 8 times, for a total of 80 pots. Inoculated seedlings were placed in a climate chamber (75% humidity; 24/16 °C, day/night temperature) at random positions, and watered 3 times per week for 12 weeks until harvest.

Table 1
Species and families of ecto-mycorrhizal (EcM) fungal isolates used in this study.

Family	Species
Hydnangiaceae	*Laccaria bicolor*
	Laccaria laccata
Russulaceae	*Lactarius rufus*
	Lactarius theiogalus
Paxillaceae	*Paxillus involutus*
Rhizopogonaceae	*Rhizopogon roseolus*
Suillaceae	*Suillus bovinus*
	Suillus granulatus
	Suillus luteus