Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates

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Abstract

Using an *in vitro* bioreactor system in which the arbuscular mycorrhizal (AM) fungus *Glomus intraradices* was grown in a soil devoid of detectable living microbes, we could show that the mycelium of this fungus contributed to the maintenance of water-stable soil aggregates and increased soil water repellency, as measured by water drop penetration time. This is to our knowledge the first demonstration of a causal link between AM fungal growth and water repellency of soil aggregates. Our results also place AM fungal contributions to soil aggregation on a firm mechanistic footing by showing that hyphae are sufficient to produce effects, in the absence of other soil biota, which have always been included in previous studies.

Keywords: Water drop penetration time; Water stable aggregates; Arbuscular mycorrhizal fungi; Soil hyphae; *In vitro* system

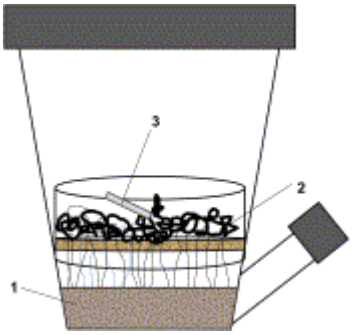
Article Outline

As widespread and abundant members of the soil biota, the obligately biotrophic arbuscular mycorrhizal (AM) fungi are a generally acknowledged biotic factor in the formation and maintenance of soil aggregates ([Tisdall and Oades, 1982], [Six et al., 2004] and [Rillig and Mummey, 2006]). Evidence for the involvement of these fungi comes from a variety of field

observational or experimental studies (Wilson et al., 2009) and controlled pot experiments in which AM fungal presence or isolate identity have been specifically modified (*e.g.*, Piotrowski et al., 2004). In none of these studies have AM fungi been present as the only component of the soil microbiota; it is generally accepted experimental practice to equilibrate microbial communities in controls and AM fungus treatments. Thus, the interaction between AM fungal hyphae and other soil biota may have contributed to the observed effects on soil aggregation. Rillig et al. (2005) documented significant effects of microbial communities associated with different AM fungal isolate cultures on soil aggregation in the absence of live AM fungi. This raises the questions: a) to what extent are microbiota associated with certain AM fungi responsible for effects on soil aggregation attributed to AM fungi?; b) is the AM fungal mycelium able to directly mediate the formation of water-stable soil aggregates (*i.e.*, without the interaction with associated microbiota)? We set out to test the latter question in the present study. Additionally, we test the hypothesis that AM fungal mycelium increases soil water repellency (Rillig, 2005); a putative factor contributing to aggregate stabilization.

We utilized a recently developed *in vitro* bioreactor system (described in detail in Gadkar et al., 2006), which was modified to include a sterilized soil/expanded clay mixture (Fig. 1). In these bioreactors (Fisher Scientific, Pittsburgh, PA), consisting of cylindrical chambers (150 mm × 100 mm) with tightly closing lids, the AM fungal culture is placed on a removable mesh (5–6 μm) assembly above the substrate without touching it. The roots are retained by the mesh and the substrate becomes the hyphal growth compartment. The soil was an Albic Luvisol from a local meadow in Berlin with the following properties: 73.6% sand, 18.8% silt and 7.6% clay; 6.9 mg/100 g P; 5.0 mg/100 g K (analyses conducted by LUFA Rostock Agricultural Analysis and Research Institute, Germany). The soil was wet-sieved to 212 μm, mixed with expanded clay (spheres up to 1 cm diam.) in a 2:1 ratio (vol:vol) to provide aeration, and 180 g of this mixture were added to each bioreactor. The bioreactors were then autoclaved (121 °C; 20 min) three times on three consecutive days. The experimental treatment consisted of presence/absence of AM fungus, with 8 replicates per treatment. We used the AM fungus *Glomus intraradices* (DAOM 197198) propagated on *Daucus carota* (wild carrot). *In vitro* cultures of this isolate were obtained from Premier Tech Biotechnologies (Rivière-du-Loup, Quebec, Canada). To the control we added non-colonized cultures. In each case, the entire intact contents of one Petri dish (9 cm dia) were added to each bioreactor. All cultures were of the same age. The bioreactors were incubated at 25 °C in the dark and the substrate was watered with 20 ml of sterile water four days after inoculation. We added 4 ml glucose (50 mg mL^{-1}) using sterile cotton rolls three times during the study, as in Gadkar et al. (2006). At each of those times, aeration was provided by opening the container for approximately 10 min in the sterile work bench. During the experiment we observed hyphal growth within pore spaces at the edge of the containers. After 12 weeks, the experimental units were destructively harvested. No visible fungal contamination was evident during the experiment in the bioreactors. Additionally, the substrate was tested for living microbial propagules by plating out aliquots on Nutrient Agar (1.5%; Sigma–Aldrich, Steinheim, Germany; N4019). No microbial growth was visible after 7–10 days. Using the ink and vinegar staining method (Vierheilig et al., 1998), AM fungal root colonization was 75% in the inoculated roots, whereas no colonization was found in the control roots. In one AM-treated bioreactor the culture failed to establish, hence $n = 7$ for this treatment.

Upon harvest the substrate was air-dried (60 °C; 48 h), and the soil, separated from the expanded clay by gently dry-sieving, used for further analyses.



Full-size image (36K)

Fig. 1. Diagram showing set-up of the modified bioreactors. The substrate mix (1) is in the bottom of the bioreactor, above which the tray (2) holding the root organ culture and AM fungal inoculum is located. The root cultures are fed with cotton rolls containing glucose (3).

We extracted extraradical hyphae using an aqueous filtration extraction method and quantified AM fungal mycelium according to the morphological criteria described by Rillig et al. (1999). We did this in the control as well, where all AM fungal hyphae will be dead (the obligate biotrophic AM fungi did not colonize host roots in the control), but – owing to the absence of a microbial community – did not decompose/disintegrate. To quantify the presence of fungal hyphae on macroaggregate surfaces, we determined the frequency of surficial hyphal presence/absence on each of 20 randomly selected aggregates (1–2 mm diam.) per sample using a stereoscope (magnification 60–80×). We used stacked sieves (0.212 mm, 0.5 mm, 1.0 mm, and 2.0 mm) to measure the soil aggregate distribution (starting mass of 50 g soil) and to calculate the mean weight diameter (Kemper and Rosenau, 1986). Additionally, to measure the amount of water-stable macroaggregates, we used a wet-sieving apparatus (sieving time 5 min; total soil used 4.0 g) as described in Kemper and Rosenau (1986), after a 10 min capillary rewetting period. Weights were corrected for coarse matter (>0.25 mm). For both the soil and water-stable macroaggregate fraction we measured the water drop penetration time (WDPT) in seconds. Using ground samples, the time to absorption of an 8 μL droplet of deionized water placed on the smoothed surface was measured for three sub-samples per bioreactor. On both the water-stable macroaggregate fraction and whole soil we also measured the percentage of total C and N using a Euro EA analyzer (HEKAtech GmbH, Wegberg, Germany). Means were compared using one-way analyses of variance.

At the end of the experiment there was about a three-fold increase in AM fungal mycelium in the inoculated bioreactors compared to the control (Table 1); additionally, a much higher proportion (5-fold) of observed aggregate surfaces carried fungal mycelium in the inoculated bioreactors compared to control. While dry-sieving revealed no differences between the treatments, using

water as a disintegrating force resulted in a significant difference, with increased water-stable macroaggregate mass in the AM fungal inoculated bioreactors. There was a trend towards an increase in soil water repellency, and there were no differences in C and N content. For the stable macroaggregate fraction (Table 1), there was a highly significant increase in water drop penetration time, while C and N content were again not different between the treatments.

Table 1.

Differences in soil parameters between sterile bioreactor systems inoculated or not with the AM fungus *Glomus intraradices*. Values are means and standard errors (n = 8 for control and n = 7 for inoculated). Significant p values (<0.05) are bolded.

Response variable	**Non-inoculated (control)**	**Inoculated**	**F value (*p* value)**
	Whole soil		
AM fungal extraradical hyphal length (m g^{-1} soil)	4.12 (0.33)	12.20 (1.12)	43.8 **(<0.0001)**
Proportion of aggregates with surficial hyphae (%)	10.0 (4.7)	47.9 (5.0)	29.4 **(0.0006)**
Mean weight diameter from dry-sieving (mm)	0.93 (0.06)	0.91 (0.05)	0.09 (0.77) ns
Water-stable macroaggregates >0.250 mm (% of soil weight)	61.8 (2.6)	72.9 (2.7)	8.75 **(0.011)**
Water drop penetration time (seconds)	9.6 (0.7)	11.9 (0.9)	4.0 (0.058) ns
Total C (%)	2.22 (0.06)	2.25 (0.06)	0.11 (0.74) ns
Total N (%)	0.19 (0.005)	0.19 (0.006)	0.11 (0.75) ns
	Stable macroaggregates		
Water drop penetration time (seconds)	12.2 (1.0)	26.3 (2.2)	37.5 **(<0.0001)**
Total C (%)	2.51 (0.03)	2.49 (0.04)	0.17 (0.8) ns
Total N (%)	0.21 (0.002)	0.21 (0.002)	1.46 (0.24) ns

There are three key points to be taken from the study: (a) we demonstrated the feasibility of using our previously described *in vitro* culture system for carrying out highly controlled, targeted studies of AM fungal mycelium effects on soil properties; and (b) we conclusively showed, to our knowledge for the first time, that AM fungi alone can be sufficient to form and/or maintain water-stable soil macroaggregates; (c) we demonstrated that AM fungal mycelium can affect soil water repellency, particularly of macroaggregates.

In vitro culture systems of AM fungi have been used successfully for an array of research topics, including systematics, fungal life cycles and structures, as well as physiological and biochemical properties of fungi and the symbiosis (Declerck et al., 2005). The use of soil medium in *in vitro* culture systems is unusual, possibly because of the difficulty to avoid contamination and the inability of direct observation of hyphae in the opaque medium; however, our completely autoclaveable container system has illustrated the potential for also using this approach to study effects on the soil substrate, with the caveat that it is impossible to conclusively prove the absence of residual microbial contamination.

While there is ample evidence for the involvement of fungi in soil aggregation in general (*e.g.* Gupta and Germida, 1988) and for AM fungi in particular, and good conceptual integration of AM effects in aggregate hierarchy theory (Tisdall and Oades, 1982), only with this study have we established clear causality for a direct AM fungal mycelium effect by excluding other players. This came, like with all mechanistic studies that employ high experimental control, at the cost of ecological realism. Carrying out greenhouse experiments on soil aggregation in which the growth medium is kept sterile is typically neither desired nor technically feasible. Even when defined AM culture material is being used in studies on soil aggregation (*e.g.*, Piotrowski et al., 2004), and care is taken to equilibrate microbial communities by adding a microbial 'wash', it should be realized that microbes could influence the observed effects. While establishing causality in terms of AM mycelium involvement in the formation of water-stable macroaggregates, our study could not address the relative importance of the interaction with soil microbiota compared to the main effects of the mycelium itself; clearly this should be a focus of future research.

Previous work has shown the existence of a link between soil hydrophobicity (for example on aggregate surfaces) and aggregate stability ([Sullivan, 1990] and [Goebel et al., 2005]). However, our study is the first to experimentally demonstrate a direct causal link between the growth of AM fungal mycelium and soil water repellency. Rillig (2005) had previously hypothesized that hydrophobins, ubiquitous proteins present in filamentous fungi but which have not yet been demonstrated to be present in AM fungi, may be responsible for some of the (sub-critical) water repellency phenomena observed in the field. Hallett et al. (2009) could, however, not find a relationship between AM fungi and water repellency in their study system. We do not know what substance(s) were responsible for the observed increase in hydrophobicity in our study. It would be exciting to utilize this bioreactor experimental system to test the idea that hydrophobins were involved; additionally, glomalin-related soil protein (GRSP; Rillig and Mummey, 2006) could also have contributed to the observed effects, even though it has likely no resemblance to hydrophobins (Gadkar and Rillig, 2006). Irrespective, with this result we can add

the mechanism of increased water repellency as a potential contribution of AM fungal hyphae to aggregate water stability.

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