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Short communication

Rotation of hyphal in-growth cores has no confounding effects on soil abiotic properties

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ABSTRACT

To disentangle effects of fungal hyphae and plant roots hyphal in-growth cores have become a common tool in research on arbuscular mycorrhizal fungi (AMF). However, it is unknown if the frequent rotation of a compartment has any side-effects that may hinder attributing findings to AMF. We set up an experiment with the presence/absence of a non-AMF microbial community, where each pot contained a rotated and a non-rotated soil core. The results show that within our rotation design soil parameters such as water content, soil structure, pH, and C and N concentrations are not influenced by the regular rotation in the absence of AMF. Our study therefore clearly underlines the validity of the rotated hyphal in-growth core as an experimental control for AMF growth and activity.

Arbuscular mycorrhizal fungi (AMF) associate with ~80% of the land plants species and are able to improve plant nutrition, soil structure and mitigate plant stress by pathogens or drought (van der Heijden et al., 1998; Rillig and Mummey, 2006; Smith and Read, 2008). As AMF are obligate biotrophic symbionts, hyphal proliferation requires a connection to a host. To disentangle the unique effects of hyphae in the absence of plant roots, hyphal in-growth cores have become a common tool to study the contribution of mycorrhizal fungi to nutrient and allelopathic compound translocation, plant growth, litter decomposition and carbon cycling (Johnson et al., 2001; Wallander et al., 2013). A cylindrical core with windows is covered with a mesh of a pore size that allows hyphae to grow inside the core but excludes the roots (usually 25–40 μm, e.g. Babikova et al., 2013; Cheng et al., 2008; Nottingham et al., 2013). One hyphal in-growth core is static while the other is rotated or moved up and down to sever the hyphae (Barto et al., 2011), and thus serves as a reduced (or non-) AMF control (Johnson et al., 2001). However, despite the broad application of this method, it is unknown if the frequent rotation of a compartment has any side-effects that may hinder attributing findings to AMF. Especially in small compartments the water content could be influenced by the repeated movement of the core, which may also have consequences for a number of processes such as decomposition, soil aggregation and nutrient transfer.

In order to test for confounding effects of the rotated core design, we set up a greenhouse experiment in either presence or absence of a natural soil microbial community, where each pot contained two hyphal in-growth cores. The soil was a loamy sand with the following properties: pH 7.1 ($CaCl_2$), 6.9 mg P/100 g soil (calcium-acetate-lactate), 0.12% N (total) and 1.87% C (total) (for analytical methods see Rillig et al. (2010)). Soil was autoclaved twice and filled in pots (3 l) where we inserted two plastic tubes (15 cm length, 32 mm diameter), which had a grid structure with 72 openings per tube of a size of 7 × 8.5 mm. The tubes (cores) were covered with a 38 μm mesh that was attached to the core with silicone glue. One soil core was left stable (static compartment), while the second soil core was rotated 3 times per week for 2–3 mm horizontally (rotated compartment) to keep the disturbance as small as possible (see also Fig. 1). Seeds of *Trifolium repens* L. were sterilized in 10% commercial bleach and sown directly into the center of the pots next to the hyphal in-growth cores. After seed emergence plants were thinned to one plant per pot. One treatment received no inoculum and was used to test for side-effects of the rotated core design ($N = 10$). For five replicate pots each plant was inoculated with a microbial filtrate of fresh field soil that was collected at a meadow of the Freie Universität Berlin. The filtrate was prepared by sieving a soil suspension through a 20 μm sieve and collecting the filtrate, thus excluding larger sized spores such as those of AMF. Half of the filtrate was autoclaved and added to non-

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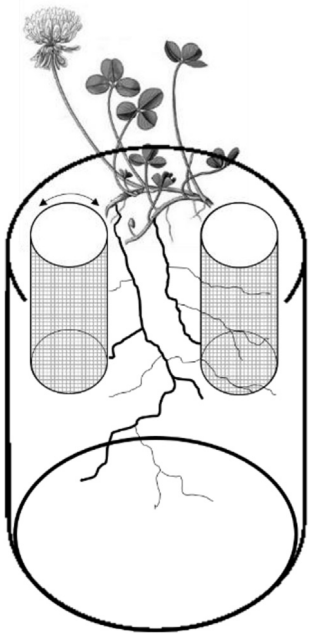

Fig. 1. Rotated core design with a 38 μm mesh: pot with plant, roots and hyphae, rotated compartment (indicated by the arrow) without roots and hyphae and static hyphal compartment (Original image of *T. repens* by Thomé, 1885).

inoculated pots. This treatment was used to test whether potential effects of the core rotation may be found in presence of natural saprobic fungi and bacteria but in absence of AMF. Additionally, we inoculated in a similar fashion another five replicates with an AMF spore extract: a soil suspension was sieved through a 38 μm sieve and what remained on the sieve was collected and surface sterilized in commercial bleach (10%). The primary purpose of this treatment was to check the success of the rotation in reducing AMF hyphal length. The greenhouse had an average temperature of 26C°/20C° (day/night). During the experiment plant leaves were cut once per week to prevent excessive growth of leaves (to reduce contamination) and roots (to avoid pot bound roots).

After 3.5 months plants and soil were harvested, dried at 40 °C, and stored at room temperature until laboratory analysis. All laboratory analyses were performed for both soil cores, except the water content was analyzed for the soil of the pot as well. Ball milled soil was analyzed for percentages of total C and N as above, as a rough indication for possible changes in basic soil properties. Soil pH and electrical conductivity (EC) were analyzed according to ISO 10390:2005 and ISO 11265:1997, respectively. Water stable soil aggregates (WSA) were assessed by wet sieving in a sieving apparatus modified after Kemper and Rosenau (1986): 4.0 g of dried soil were rewetted by capillary action and sieved for 5 min using a 250 μm sieve. We separated the coarse matter by crushing the aggregates that remained on the sieve and pushing the soil through. Coarse matter and soil were collected and dried at 80 °C for 36 h. We calculated the amount of total WSA and corrected the calculations for coarse matter. Hyphae were extracted from 4.0 g of soil (Jakobsen et al., 1992), stained with Trypan blue, and hyphal length ($m \cdot g^{-1}$) soil was measured according to Rillig et al. (1999). Additional to the total extractable hyphae we estimated the length of AMF and non-AMF hyphae. Dark- to light-blue stained aseptate hyphae with characteristic unilateral angular projections ("elbows and coils") were considered mycorrhizal (Mosse, 1959), whereas non blue stained or blue stained hyphae with regular septation or straight growth were considered non-mycorrhizal. Very short or deteriorated pieces were excluded from the analysis.

Water content of the soil was analyzed gravimetrically by weighing at harvest and after drying. All statistical analyses were conducted using the software R, version 3.0.2 (R Core Team, 2013). Soil parameters were analyzed by linear mixed-effects models with the pot number as random factor using the package 'nlme' (Pinheiro et al., 2013).

Our results show that in the absence of AMF (i.e. in the non-inoculated control pots) there was only slight variation in the measured abiotic soil parameters between the rotated and static compartment (see Table 1). The only significant difference was a slightly lower EC in the rotated cores in the microbial filtrate treatment, which we will discuss below. This suggests that – within our rotation regime – the design is generally suitable for studying specific effects of AMF without confounding abiotic effects. However, compared to the cores, the water content was significantly lower in the rest of the pot (10.2 ± 2.0%; $p = 0.001$), indicating that water was removed by the roots and that the mesh may have obstructed the water movement between the pot and the soil cores. Such an effect could be strengthened by a more severe rotation regime than we applied here, and could cause differences to arise between rotated and static cores even though they were absent in our experiment. In the study by Johnson et al. (2001) for instance, where the hyphal in-growth cores were presented for the first time, the cores were rotated by 45 ° around the vertical axis every week. In other designs rotation was as much as 180 ° once per week or two complete turns twice a week (Nottingham et al., 2013; Weremijewicz and Janos, 2013). The latter represent a considerable disturbance that might cause comparatively stronger effects of the rotating such as the creation of air gaps between the core and surrounding soil, which might hamper the movement of water and nutrients.

As can be seen in Table 1, rotating cores successfully reduced the length of total extractable hyphae, and in case of the treatment with AMF spore inoculation putative AM hyphae strongly contributed to this effect. However, following our criteria for identification of non-AMF hyphae we could also observe a reduction of non-AM fungal hyphae in the rotated cores. These filamentous fungi might have entered the soil with the spore extract, and in the case of the non-inoculated treatment as airborne propagules. The presence of these fungi is therefore unsurprising, as greenhouse experiments generally do not remain sterile. This does however indicate that processes within this experimental design attributed to AMF in the past might have been partly caused by saprobic fungi, at least to the extent that they are affected through rotating cores as we show here. This effect of core rotation on non-AMF may be related to the formation of branched networks that are also severed, which reduces their proliferation efficiency (Barto et al., 2011).

The only parameter that was significantly different between compartments in our study was the electrical conductivity in the treatment with microbial filtrate (see Table 1). The conductivity was higher in the static core, suggesting that the hyphae (more abundant in the static core; Table 1) retained some of the ions, possibly directly within their biomass or indirectly through the stimulation of associated microbes that incorporated those ions, which were then released upon soil drying and measurement of EC (Singh et al., 1989). Given the relative subtlety of the effect of rotating on EC, and the fact that it only occurred in the presence of a soil microbial filtrate, we believe it is unlikely to compromise the rotated core design for attributing effects to AMF. In our experiment hyphae identified as non-AMF tended to be reduced in the treatments inoculated with AMF, which would make any slight modifications in soil properties not caused by AMF even less severe, as AMF are generally found to have stronger effects on these same

Table 1
Soil analyses of the non-inoculated treatment, the treatment with microbial filtrate, and the treatment with AMF and microbial filtrate. Values represent means and standard errors in parentheses, the *p*-value refer to the null-hypothesis that rotated and static cores are the same within treatment, $N = 10$ for the non-inoculated treatment, and $N = 5$ for the two other treatments.

	Non-inoculated treatment			Treatment with microbial filtrate			Treatment with AMF and microbial filtrate		
	Rotated	Static	*p*-value	Rotated	Static	*p*-value	Rotated	Static	*p*-value
All extracted hyphae ($m \cdot g^{-1}$)	1.44 (0.05)	2.41 (0.10)	**<0.001**	1.51 (0.11)	3.13 (0.14)	**0.001**	1.62 (0.13)	5.28 (0.21)	**<0.001**
AMF hyphae ($m \cdot g^{-1}$)	0.30 (0.03)	0.35 (0.03)	0.28	0.28 (0.05)	0.31 (0.03)	0.65	0.43 (0.03)	3.01 (0.19)	**0.005**
Non-AMF hyphae ($m \cdot g^{-1}$)	1.14 (0.06)	2.07 (0.09)	**<0.001**	1.23 (0.09)	2.83 (0.14)	**<0.001**	1.19 (0.11)	2.27 (0.10)	**0.009**
Water content (%)	12.8 (1.6)	13.6 (1.7)	0.23	11.6 (2.2)	11.2 (2.3)	0.39	9.2 (0.9)	8.8 (0.6)	0.48
EC ($\mu S \cdot cm^{-1}$)	346 (5)	347 (8)	0.98	313 (13)	338 (7)	**0.03**	311 (10)	334 (11)	**0.01**
pH ($CaCl_2$)	6.20 (0.02)	6.16 (0.01)	0.19	6.03 (0.01)	6.03 (0.01)	0.69	6.08 (0.01)	6.09 (0.02)	0.68
C (%)	3.20 (0.13)	3.29 (0.21)	0.67	2.80 (0.63)	2.82 (0.67)	0.93	3.29 (0.83)	3.38 (0.44)	0.85
N (%)	0.26 (0.01)	0.26 (0.02)	0.75	0.23 (0.06)	0.23 (0.06)	0.85	0.27 (0.07)	0.26 (0.04)	0.89
WSA (%)	65.0 (3.7)	64.0 (4.0)	0.7	51.4 (2.3)	59.4 (3.4)	0.09	59.0 (4.5)	59.4 (3.4)	0.59

$P < 0.05$ in bold.

properties (e.g. Bago et al., 1996; Johnson et al., 2001; Barto et al., 2011; Babikova et al., 2013) (See also Table 1). In conclusion, our results clearly underline the validity of the rotated hyphal ingrowth core, as pioneered by Johnson et al. (2001), as an experimental control for AMF growth and activity.

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References

Babikova, Z., Gilbert, L., Bruce, T.J.A., Birkett, M., Caulfield, J.C., Woodcock, C., Pickett, J.A., Johnson, D., 2013. Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. Ecology Letters 16, 835–843.

Bago, B., Vierheilig, H., Piché, Y., Azcon-Aguilar, C., 1996. Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices* grown in monoxenic culture. New Phytologist 133, 273–280.

Barto, E.K., Hilker, M., Mueller, F., Mohney, B.K., Weidenhamer, J.D., Rillig, M.C., 2011. The fungal fast lane: common mycorrhizal networks extend bioactive zones of allelochemicals in soils. Plos One 6.

Cheng, X.M., Euliss, A., Baumgartner, K., 2008. Nitrogen capture by grapevine roots and arbuscular mycorrhizal fungi from legume cover-crop residues under low rates of mineral fertilization. Biology and Fertility of Soils 44, 965–973.

ISO 10390, 2005. Soil Quality – Determination of pH.

ISO 11265, 1997. Soil Quality – Determination of the Specific Electrical Conductivity.

Jakobsen, I., Abbott, L.K., Robson, A.D., 1992. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. 1. Spread of hyphae and phosphorus inflow into roots. New Phytologist 120, 371–380.

Johnson, D., Leake, J.R., Read, D.J., 2001. Novel in-growth core system enables functional studies of grassland mycorrhizal mycelial networks. New Phytologist 152, 555–562.

Kemper, W.D., Rosenau, R.C., 1986. Methods of soil analysis. Part I. Physical and Mineralogical Methods.

Mosse, B., 1959. Observations on the extramatrical mycelium of a vesicular-arbuscular endophyte. Transactions of the British Mycological Society 42, 439–448.

Nottingham, A.T., Turner, B.L., Winter, K., Chamberlain, P.M., Stott, A., Tanner, E.V.J., 2013. Root and arbuscular mycorrhizal mycelial interactions with soil microorganisms in lowland tropical forest. FEMS Microbiology Ecology 85, 37–50.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., R-core, 2013. Linear and Nonlinear Mixed Effects Models, Version 3.1-111.

R Core Team, 2013. R: a Language and Environment for Statistical Computing. Vienna, Austria. http://www.R-project.org.

Rillig, M.C., Field, C.B., Allen, M.F., 1999. Soil biota responses to long-term atmospheric CO2 enrichment in two California annual grasslands. Oecologia 119, 572–577.

Rillig, M.C., Mummey, D.L., 2006. Mycorrhizas and soil structure. New Phytologist 171, 41–53.

Rillig, M.C., Mardatin, N.F., Leifheit, E.F., Antunes, P.M., 2010. Mycelium of arbuscular mycorrhizal fungi increases soil water repellency and is sufficient to maintain water-stable soil aggregates. Soil Biology & Biochemistry 42, 1189–1191.

Singh, J.S., Raghubanshi, A.S., Singh, R.S., Srivastava, S.C., 1989. Microbial biomass acts as a source of plant nutrients in dry tropical forest and savanna. Nature 338, 499–500.

Smith, S.E., Read, D.J., 2008. Mycorrhizal Symbiosis, second ed. Academic Press, San Diego and London. 605 pp.

Thomé, O.W., 1885. Flora von Deutschland, Österreich und der Schweiz, Gera (Germany).

van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, A., Sanders, I.R., 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature 396, 69–72.

Wallander, H., Ekblad, A., Godbold, D.L., Johnson, D., Bahr, A., Baldrian, P., Bjork, R.G., Kieliszewska-Rokicka, B., Kjoller, R., Kraigher, H., Plassard, C., Rudawska, M., 2013. Evaluation of methods to estimate production, biomass and turnover of ectomycorrhizal mycelium in forests soils – a review. Soil Biology & Biochemistry 57, 1034–1047.

Weremijewicz, J., Janos, D.P., 2013. Common mycorrhizal networks amplify size inequality in *Andropogon gerardii* monocultures. New Phytologist 198, 203–213.