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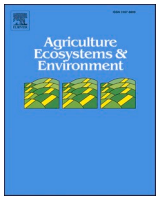

Linking stable soil carbon and microbes using rapid fractionation and metagenomics assays – First results screening fungal inoculants under wheat crops

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ABSTRACT

Increasing soil carbon in agricultural systems can help mitigate and eventually reverse climate change. Soil microorganisms play a key role in regulating soil carbon accrual and stability. Questions remain about the link between microbes and soil carbon outcomes and how to leverage microbial processes. Here we screen microbial inoculation (endophytic fungal isolates) regarding their effects on soil carbon in a wheat pot trial using a rapid soil carbon fractionation assay and link the results with microbial community structure and function observations. Under the specific chemical and biological conditions of the plant-soil-environmental system, two of the 17 fungi tested increased soil carbon in close proximity to the roots by ~15 %. This increase was associated with the medium stable, soil aggregate organic matter fraction (up to +21 %) and also mineral-associated organic matter, the long-term soil carbon storage (+10 %). Some of these changes were linked to a shift in predicted functional genes (whole metagenome, long read sequencing) and an increase in bacterial and fungal biomass (phospholipid fatty acid analysis). Microbial inoculation did not induce a statistically significant shift in the microbial composition (metagenomics), which, instead, correlated with the labile, particulate organic matter pool. While it is unclear whether the two endophytes directly influenced soil carbon cycling or had an indirect effect, through altering existing microbial processes, it demonstrates their potential for positive impacts on soil carbon that needs confirming in field trials. The combination of high throughput assays we present here could further help link carbon stability with microbial indicators and build more accurate soil carbon models.

1. Introduction

Carbon (C) sequestration in the form of soil organic carbon (SOC) is crucial for reducing atmospheric carbon dioxide levels and stabilising the climate (Paustian et al., 2016; Smith, 2016). SOC, present in soil as soil organic matter (SOM), has a positive environmental and agricultural impact. It reduces nutrient leaching and increases water retention (Kopittke et al., 2021; Smith, 2016) and SOM contents are positively correlated with crop yields globally (Oldfield et al., 2019). To benefit from these effects long-term and to influence atmospheric carbon dioxide levels on a societal relevant scale, SOC needs to be stored in a stable form to avoid re-release of C on short-term.

Carbon in the form of mineral-associated organic matter (MAOM) is generally considered a long-term SOC reservoir (Hemingway et al., 2019). Most researchers agree that the least stable SOC pool is carbon present as particulate organic matter (POM), which is mostly derived from plant litter and roots that are in their early stages of decomposition (Abramoff et al., 2018; Cotrufo et al., 2019; Lavallee et al., 2020; Poeplau et al., 2018). The persistence of SOC is also linked to soil structure due to physical protection (occlusion) of POM from microbial decomposition within soil aggregates (Peng et al., 2017; Rovira and Greacen, 1956; Six and Paustian, 2014). The carbon within this aggregate OM fraction (AggOM), which besides POM also contains mineral surfaces and hence associated MAOM, is considered a medium-term stable SOC pool (Abramoff et al., 2018; Poeplau et al., 2020, 2017). Newer SOC process models, e.g., the Millennial model, use these three main SOC pools and add dynamic turnover stocks, such as carbon in dissolved organic matter and microbial biomass C to model and predict C flows (Abramoff et al., 2018, 2022). So far, however, it is a major challenge to include microbial composition as mediator of SOM turnover in such

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models. This study makes an attempt at empirically linking microbial composition with different soil carbon fractions.

Microorganisms are responsible for the respiration of plant C, but they also convert C into more stable SOC forms (Liang et al., 2019). Plant C delivered below ground through rhizodeposits is converted into stable SOC much more efficiently than from plant litter that decomposes on the soil surface (Sokol and Bradford, 2019). However, a large proportion of gross rhizodeposition, on average ~55 %, is still decomposed and released back as CO_2 (Pausch and Kuzyakov, 2018). Therefore, increasing the retention of plant rhizodeposits in soil in the form of AggOM and MAOM through management of microbial communities in the plant rhizosphere could be a way to increase C sequestration in agricultural systems (Kallenbach et al., 2019), while also prodiving benefits on nutrient cycling and water retention. There is increasing evidence that specific microbial traits foster microbial carbon accrual (Sokol et al., 2024; Tao et al., 2023; Whalen et al., 2024) and there might be potential to utilise such microbes for targeted alterations of the soil microbiome for better soil C outcomes.

Root-associated fungi that live within and around the roots (rhizosphere) can influence both processes of SOC formation and decomposition and hence the total SOC stock (Soudzilovskaia et al., 2019; Wilson et al., 2009). Much research has been conducted on the effects of mycrorrhizae as drivers of SOC formation (Frey, 2019). However, non-mycorrhizal, endophytic fungi, fungi that spend all or parts of their life within plant tissue (endosphere), are as ubiquitous, can be beneficial for plants in times of water stress and show characteristics with important implications for SOC dynamics (Hardoim et al., 2015; Rodriguez et al., 2009; Stuart et al., 2024). Although root endophytes are often distinguished from mycorrhizae, they comprise a large, diverse group of fungi and a clear definition is lacking (Hardoim et al., 2015; Rodriguez et al., 2009; Vandenkoornhuyse et al., 2015). Since endophytes can also spend only part of their life cycle within the plant roots and other parts as free-living organisms in the rhizosphere, they can modify the biochemistry of both endo- and ectosphere (Canarini et al., 2019; Gavito et al., 2019; Laheurte et al., 1990).

Plant-associated microorganisms, such as mycorrhizal fungi, constitute a strong sink for plant rhizodeposits (Canarini et al., 2019; Gavito et al., 2019) and hence could increase SOC by acquiring plant C and depositing it in soil. Specific fungi can further increase the conversion efficiency of plant C (exudates) into fungal biomass and subsequent necromass and SOC compared to existing microbial communities, lowering plant-C losses (Anthony et al., 2020; Kallenbach et al., 2019, 2016). The role of endophytic fungi in C cycling, however, is poorly investigated. A few studies show successful increases in SOC content using endophytic fungal isolates, yet mechanisms (direct vs. indirect inoculation effects) and the link with SOC of different stability are not well understood (Alves et al., 2021; Mukasa Mugerwa and McGee, 2017; Stuart et al., 2024).

Here we screen 17 endophytic fungi regarding their effect on total SOC content. We subsequently analyse SOC fractions of different stability in treatments that successfully increased total C content to link these fractions with shifts in the soil microbiome. The objectives of this study were to (i) evaluate a novel, rapid soil fractionation approach that is linked with a soil metagenomics approach, (ii) assess the effects of endophytic fungal isolates on SOC content and SOC fractions using this pipeline, and (iii) investigate relationships between changes in SOC of different stability with changes in microbial biomass, composition and functional genes. We hypothesize that if C gains are observed they will occur in the AggOM fraction where fungi carbon would be deposited initially, and such changes would be related to increased fungal biomass.

2. Materials and methods

2.1. Pot trial

The soil used in the trial was a red Luvisol taken from the top 20 cm of an agricultural field in central western New South Wales in Australia (Young). It had a starting total soil C content of 1.1 %, pH of 7.34 and a total nitrogen (N) content of 0.20 %. Details of the soil can be found in S1 Table.

The soil was sieved to < 10 mm to homogenise but to avoid disturbing the soil structure and filled into 11 cm diameter and 11 cm high, round pots (~1.2 kg of soil). Endophytic fungi, isolated from the field from grasses and shrubs from diverse natural environment across Southeastern Australia, were grown on potato dextrose agar plates. They were initially screened evaluating the successful colonization of plants (including wheat) and examining potential soil property responses to inoculation as described previously (Stuart et al., 2024). Overall, 17 fungal isolates were tested and supplied by SoilCQuest / Loam Bio Pty Ltd (Orange, New South Wales, Australia). Of the fungi inoculants 14 were attributed to the phylum Ascomycota, two to the phyla Mucoromycota and one to the phylum Basidomycota. The selected species belong to the most common occurring phyla of endophytic fungi found in nature (Li et al., 2025). In S2 Table information on family level for all fungi is shown.

Small agar-fungi plugs were cut (5 mm×5 mm x 2 mm) and one fungi plug was placed in a 20 mm deep depression in the soil of each pot. This added ~0.0005 g C to the soil in each treatment, compared to ~13.2 g of SOC in each pot (0.004 % C added). All inoculated pots received the same amount of agar. Wheat seedlings (*Triticum aestivum*, Condo variety) were pre-germinated for 3 days in darkness (24 hour in the fridge at 4°C, followed by 48 hours in the lab at 20°C), and one seedling per pot was transplanted over the fungal plug at the same time when the fungi was added.

The plants were grown in a growth chamber without fertiliser addition and a randomised block design was used for eight replications per treatment including eight planted controls without fungal inoculation (144 pots in total). The temperature and light profiles were adjusted following the actual conditions of the winter growing season of 2018 (May-November) in Parkes, New South Wales, Australia with a temperature profile of ~8–10°C at night and ~18–25°C during the day. The pots were watered regularly (~every 3 days) using tap water. After four months, the wheat plants were fully matured and senesced and were cut above the soil level, dried at 105° for 24 hours and weighed to determine aboveground plant biomass.

2.2. Soil sampling and total C analysis

Soil cores were taken with 50 mL centrifuge tubes underneath the plant stem to a depth of ~3 cm. The soil was dried at 60°C for 3 days, followed by gentle tapping with a pestle in a mortar and sieved to < 2 mm to remove gravel and larger plant roots. The roots took in most of the space within the pot, therefore, we refer to this sample as rhizosphere soil.

A subsample (~400 mg) was ground, and the contents of C and N were determined with a VarioMax 3000 (Elementar, Germany) (peak anticipated N: 210 s, oxygen dosing time: 15 s, oxygen dosing: 70 mL min^{-1}, furnace temperatures: 900°C, 900°C and 830°C; helium as carrier gas).

After screening for total C content, the samples with significant changes in total C content (treatments with fungi 112 and 1852), including the control, were chosen for further analysis. An (unground) sample was fractionated using the protocol described in next section.

2.3. Three-pool soil C fractionation assay

While it is understood that soil C occurs as continuum of decomposition products (Lehmann and Kleber, 2015), soil C fractionation methodologies allow to separate soil C into conceptually defined pools that are related to different degrees of stability and protection. The soil fractionation assay used here assesses soil stability based on protection of C (in the form of SOM) by its environment, i.e., physical protection in

soil aggregates (AggOM) and protection through sorption to minerals (MAOM). Many studies use simple two-pool carbon fractionation methods that differentiate MAOM and POM (Jiang et al., 2017; Liu et al., 2025; Pierson et al., 2021), however, this simplification does not accurately reflect natural protection mechanisms of SOC, and the harsh initial disaggregation that is part of these procedures leads to breaking up of POM and overestimation of MAOM (Cotrufo et al., 2019). The AggOM pool has shown medium-term carbon permanence and differentiate between physical and chemical carbon protection mechanisms, which is important given conservation farming practises specifically target soil aggregates stabilisation (Abdalla et al., 2016; Blanco-Canqui and Lal, 2004; Buss et al., 2024, 2023; Golchin et al., 1995; Oades, 1984). Therefore, we chose a method that fractionates into POM, MAOM and AggOM.

We modified a method that was previously evaluated by Poeplau et al., who compared 20 different methods (Poeplau et al., 2018). They concluded that dispersion of aggregates followed by size fractionation and a subsequent density separation yielded the best results (Poeplau et al., 2018). We aimed to develop a cost-effective and rapid approach to enable analyses of large number of samples in follow-up experiments, so various adjustments were performed allowing for higher throughput analysis. These adjustments are outlined below and include using molecular sieves that fit onto 50 mL centrifuge tubes avoiding the use and cleaning of full-sized sieves to accelerate sample processing to ~50 samples per day.

We also added a step to determine chemical recalcitrance, so inherent stability of soil C. More details on the rationale of selecting this method is described in the Supplementary Information. A flow-chart is presented in Fig. 1.

Samples of the treatment with a statistically significant increase in total soil C contents in the rhizosphere (24 samples in total) were fractionated without prior soil grinding to keep soil aggregates intact (AggOM). The following steps were performed: (i) 10 g of soil (dried at 40°C) was shaken with 25 mL deionised water and 2 glass beads (diameter 16 mm) for 5 min at 80 rpm in 50 mL falcon tubes on an orbital shaker with the falcon tubes lying horizontally; (ii) wet sieving was performed using molecular sieves of 70 µm size that fit onto 50 mL falcon tubes (pluriStrainer, pluriSelect, Leipzig, Germany), which accelerated the sieving process (since our method uses less water than comparable wet-sieving methods and hence separation of soil particles is less complete, a larger sieve size than the typical 50, 53 or 63 µm was used to get comparable results to full wet-sieving with higher amounts of water but smaller sieve sizes); (iii) the soil left on the sieve was washed back into the falcon tube with 12.5 mL of deionised water and the tube was vortexed for 2 s, then the sample was sieved again and the process was repeated, which resulted in a total water-to-soil ratio of 5:1 (50 mL of water to 10 g of soil); (iv) for separating POM and AggOM sodium iodide (NaI) adjusted to a density of 1.8 g cm^{-3} was used as recommended in Sohi et al. (Sohi et al., 2001), who tested 1.6, 1.7 and 1.8 g cm^{-3}, and which is also used in the soil C model MEMS (Robertson et al., 2019); (v) the samples were centrifuged at 3000 rpm for 10 min (Allegra X-12R; Beckman Coulter, Brea, United States) to ensure the AggOM fraction remains at the bottom of the tube; (vi) the POM and AggOM fraction were separated by decanting the content of the tube onto a Whatman No. 2 filter paper through turning the tube by 360° while decanting; (vii) the filter paper was washed with deionised water, dried and weighed to determine the POM fraction; (viii) to remove sodium iodide residues, the AggOM fraction was washed with deionised water, though only once to avoid C loss (Plaza et al., 2019) (the washing step reduces mass recovery from a mean of 117 % to 97–101 %); (ix) the MAOM fraction (derived from the sieving step; <70 µm) was separated from the dissolved organic matter fraction via centrifugation at 3000 rpm for 30 min (instead of using time-intensive vacuum filtration to <0.45 µm) as suggested previously (Robertson et al., 2019).

The POM, AggOM and MAOM fractions were dried, weighed and the total C and N contents were determined via combustion analysis (details about analysis above). The data were expressed as C and N in weight percent that is associated with the respective soil fraction.

In addition to the physical fractionation assay, which assesses the protection of soil C from degradation because of its environment, we also conducted a chemical recalcitrance test, which assesses the inherent stability of soil C. The test "mimic[*s*] strong enzymatic decay and isolate the oxidation-resistant soil C fraction" as outlined in Poeplau et al. (2017). As to Poeplau et al. (2017), we used 6 % sodium hypochlorite

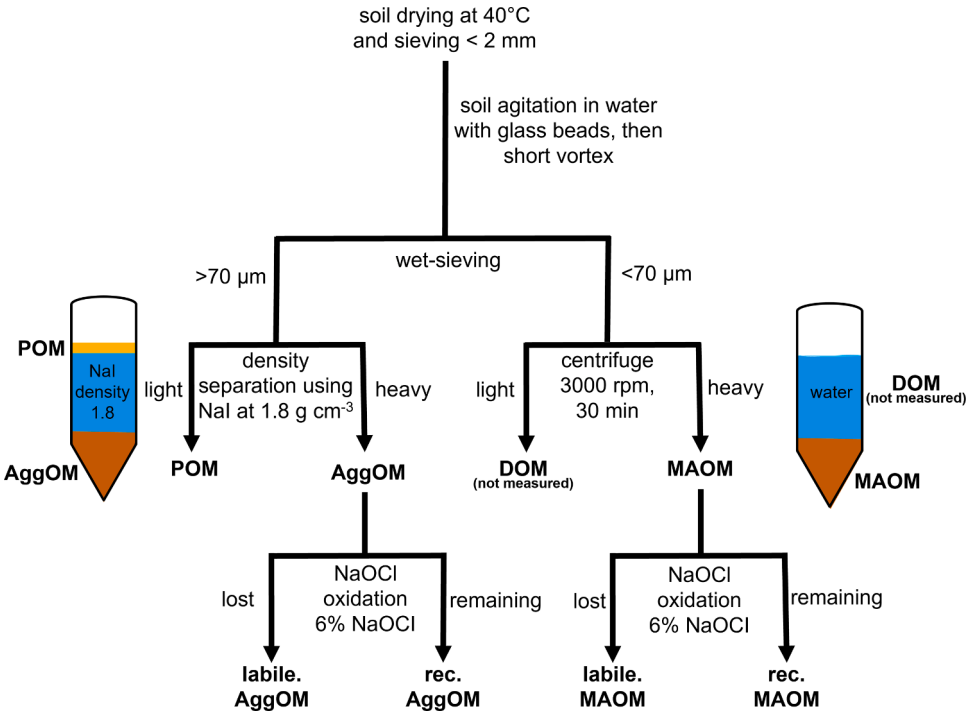

Fig. 1. Schematic of soil carbon fractionation protocol developed and used in this study.

(NaOCl) that was added to 1 g of the dried AggOM and MAOM fractions. The samples were incubated in the lab at room temperature for ~20 hours and subsequently centrifuged at 3000 rpm for 10 min. In contrast to Poeplau et al., who investigated high-SOC soils (4–6 % C), we only conducted one sodium hypochlorite (NaOCl) incubation (further incubations did not decrease C levels in our ~1 % C-containing soils). The NaOCl solution was decanted, and the treated soil was washed once with deionised water, followed by centrifugation and decanting. Finally, the samples were weighed, and the remaining C content was measured.

2.4. Soil metagenomics

For our soil metagenomics approach, we used a commercial DNA extraction kit (DNeasy PowerSoil Pro Kit, Qiagen, Hilden, Germany) and portable sequencers (Flongle; Oxford Nanopore Technology, Oxford, UK, US$10 per sample). This approach does not need investment in equipment allowing for flexible, rapid and low-cost in-house testing.

An oven dried sub sample (400 mg soil) was taken from the ground rhizosphere soil samples and subjected to DNA extraction using the DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) using the manufacturer's instructions. The DNA concentration in each sample was measured using a ND-1000 spectrophotometer (NanoDrop; Thermo Fisher Scientific, Waltham, United States) using 1 μL of liquid. Subsequently, the DNA was barcoded using the Oxford Nanopore rapid barcoding kit following manufacturer's instructions using 1 μl of rapid barcode and eluting with water. The 24 samples were pooled in 2 groups of 12 samples and each run on a Flongle sequencer (Oxford Nanopore Technology, Oxford, UK). Various studies have proven that metagenomic sequencing, particularly ONT Oxford Nanopore Technology, can be used for accurate and rapid identification of microbial species within environmental samples (Hamner et al., 2019; Loit et al., 2019; Petersen et al., 2020).

Data obtained from the Flongle sequencer was converted into DNA sequences (basecalled) and demultiplexed with Guppy (version: 5.0.7; Oxford Nanopore Technology, Oxford, UK). Subsequently, sequence libraries were curated, removing all short (<50 bp) and low quality (<q7) sequences using NanoPack (De Coster et al., 2018). The data is deposited on NCBI (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1091785).

2.4.1. Microbial composition

Curated sequence libraries were blasted against the NCBI nucleotide database version 5 (downloaded on 2021–09–30) (Sayers et al., 2021). Finally, the taxonomic ID for the single best blast hit per sequence was extracted and using the python3 library "The Environment for Tree Exploration" (Huerta-Cepas et al., 2016), the full taxonomic classification (phylum, class, order, family, genus, and species) of each sequence match was obtained. Soil samples with low sequence quality (less than 300 different microbial species) were filtered out (data quality not high enough / not reproducible). Furthermore, no match sequences and sequences that were assigned to species that were only detected once in a sample were filtered out. Sequences matching a phylum observed in only a single sample were also filtered out.

2.4.2. Functional genomics

Metagenome functional annotation was performed by eggNOG (version: 5.0) (Cantalapiedra et al., 2021) using the sequence aligner DIAMOND (version: 2.0.15) (Buchfink et al., 2021). Within each sample, eggNOG identified homologues to known gene sequences and subsequently grouped the identified genes within functional categories and metabolic pathways. Using these functional annotations KEGG Orthology terms were extracted and used for statistical analysis (see details below). No match sequences and sequences that were assigned to genes that were only detected once in a sample were filtered out. Sequences matching a gene observed in only a single sample were also filtered out.

2.5. Phospholipid fatty acid (PLFA) analysis

Microbial phospholipid-derived fatty acids (PLFA) in soils were extracted from 2 g of dried soil following the high throughput method developed by Buyer and Sasser (2012). Briefly, soil was extracted with methanol-chloroform-phosphate buffer (2:1:0.8 in volume), then fractionated into lipid types with a silica gel column followed by mild alkaline methanolysis to produce phospholipid fatty acid methyl esters (PLFAME). PLFAME were dried and analysed for composition and quantification of concentration relative to an internal standard via gas chromatography. Gas chromatography was performed on an Agilent 7890 A GC (Agilent Technologies, Wilmington, DE, USA) and FAME profiles were identified using the MIDI PLFAD1 calibration mix and the software SHERLOCK version 6.2 (MIDI, Inc., DE, USA). The abundance of individual PLFA was calculated as ug PLFA g^{-1} dry soil. PLFAMEs association as markers for different microbial groups was followed according to Joergensen (2022).

2.6. Statistics

For the statistical analysis and data visualisation SigmaPlot 11.0 (SYSTAT Software Inc) and R studio version 2022.07.1 were used. Raw data can be found in the Supplementary Information Excel File.

Outliers (extreme points) were removed if outside three times the interquartile range (R function identify_outlies) (only one total C value in the control treatment was removed). Data on total C content in the rhizosphere soil of all treatments (Fig. 2) and fractionation (Fig. 3), functional gene abundance (S5 Table) and PLFA data (Fig. 7) of the control and the two samples with increased total soil C content were analysed via one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test that compares fungi treatments to the control. A significance level of < 0.05 was used.

For correlating microbial composition and PLFA data with soil C fractions, the Spearman Rank correlation test was used (R function ggscatter) and coefficient and p-value reported within the figures (e.g., Fig. 5).

Shannon diversity index on phyla level microbial count data was run using the DescTools package in R. Non-Metric Multi-Dimensional

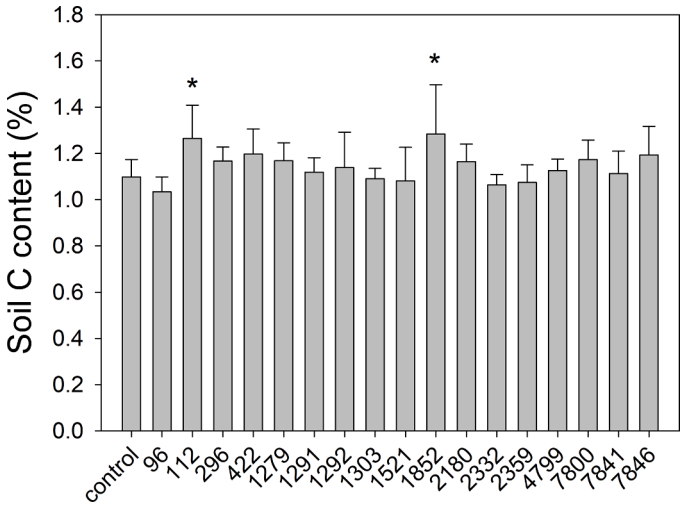

Fig. 2. Total C content in soil rhizosphere at harvest as affected by 17 fungal endophytes. Mean and standard deviation (SD) of eight replicates reported. Asterisks mark statistically significant differences determined by one-way ANOVA followed by Dunnett's post hoc test comparing fungi treatments to the controls ($p < 0.05$). ANOVA: $p = 0.00012$, Dunnet's 112-control: $p = 0.032$, Dunnet's 1852-control: $p = 0.012$. Kruskal Wallis test: $p = 0.00067$; Dunn test with Benjamini and Hochberg adjustment 112-control: $p = 0.067$, control-1852: $p = 0.090$.

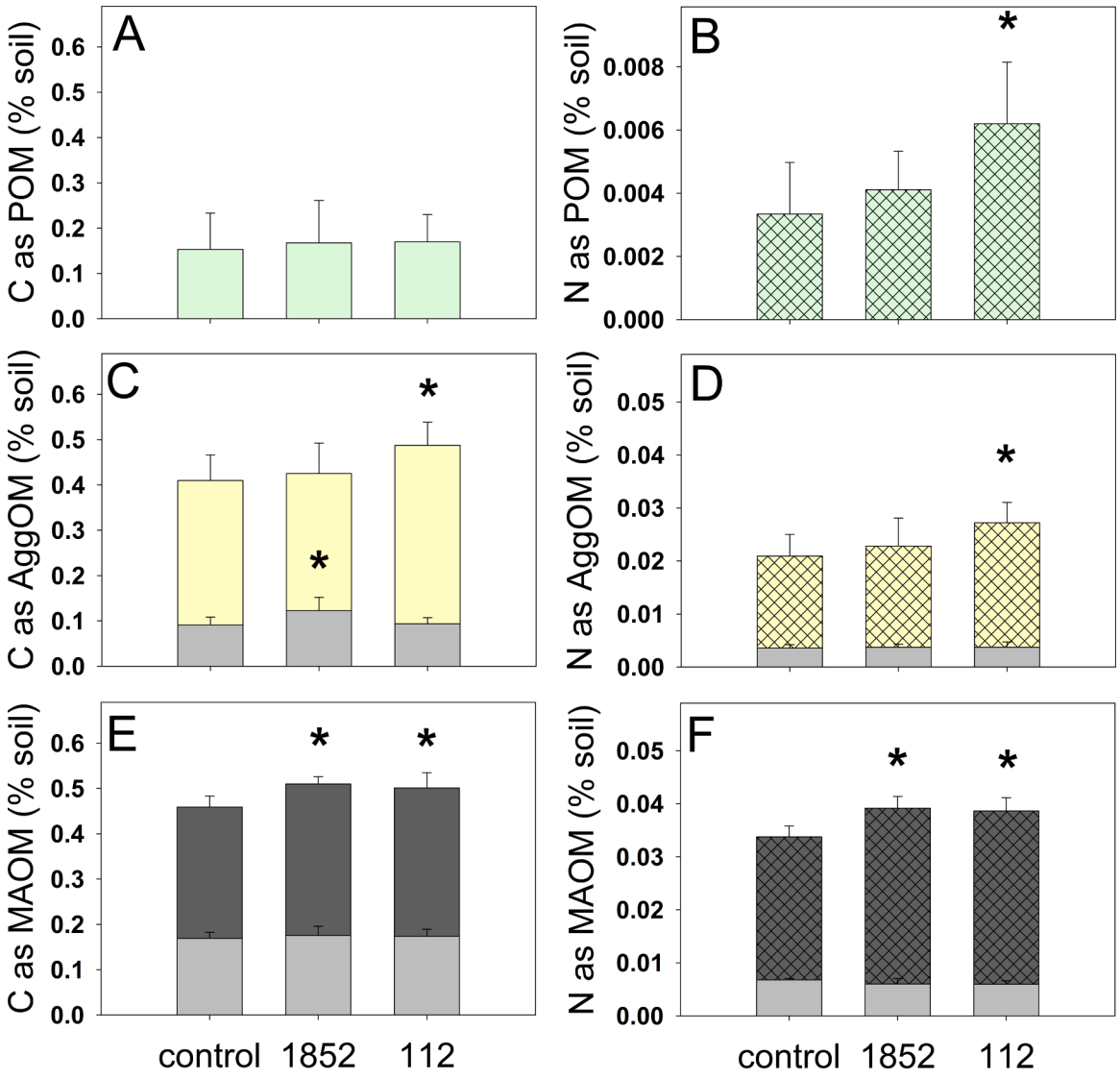

Fig. 3. Contents of carbon (A, C, E) and nitrogen (B, D, F) in soil (%) present as (A,B) particulate organic matter (POM), (C, D) aggregate organic matter (AggOM) and (E,F) mineral-associated organic matter (MAOM) in control and two fungal isolates (1852, 112). Recalcitrant carbon (and nitrogen) in the respective AggOM and MAOM fractions determined via chemical recalcitrance test (bleach oxidation) are highlighted by grey bars in C to F. Mean and standard deviation (SD) of eight pot replicates reported. Asterisks mark statistically significant differences determined by one-way ANOVA followed by Dunnett's post hoc test comparing fungi treatments to the controls ($p < 0.05$).

Scaling (NMDS) was performed using raw count phyla abundance and functional gene data (based on KEGG Orthology) (vegan package; metaMDS; Bray-Curtis dissimilarity matrix) and NMDS 1 and 2 were subsequently plotted. Samples with low sequence quality (<300 species per sample) were considered outliers and removed from the analysis.

In addition, a Permutational Multivariate Analysis of Variance (PERMANOVA) was conducted (on raw count data) to determine significant differences in microbial composition and functional genes among the treatments (Martinez Arbizu, P. (2020). pairwiseAdonis: Pairwise multilevel comparison using adonis. R package version 0.4; permutations: 9999). Differences among taxonomic groups and functional genes between the treatments were additionally determined via ANOVAs on the centre log transformed compositional data, followed by Dunnett's post-hoc test.

Using the Mantel test approach (mantel function, vegan package) raw count data of microbial abundance and functional genes (converted into Bray-dissimilarity matrix using vegdist function of vegan package in R) were correlated with soil C fractionation data (converted into Euclidean distances using dist function in R) with Pearson correlation and 9999 permutations. With the envfit function soil C fractionation data (converted into vectors) with a significant effect (based on Mantel test) were overlaid with NMDS results (Figs. 4A and 5) based on linear fit of the variables.

3. Results

3.1. Screening for changes in soil C content due to fungal endophytes

In the control treatment, the soil C content in the rhizosphere did not change over the course of the experiment (soil C content 1.1 %). However, isolates 112 and 1852 significantly increased the C content by 15 % and 17 % compared to the control, respectively, tested via one-way ANOVA followed by Dunnett's test (Fig. 2; ANOVA model output in the SI). The residuals of soil C data were not normality distributed (Shapiro-Wilk test) but soil C did show equal variances among the treatments (Levene test). Despite the robustness of ANOVA against violation of the normality assumption (Schmider et al., 2010), we also performed a Kruskal Wallis test ($p = 0.00067$) that we followed with a

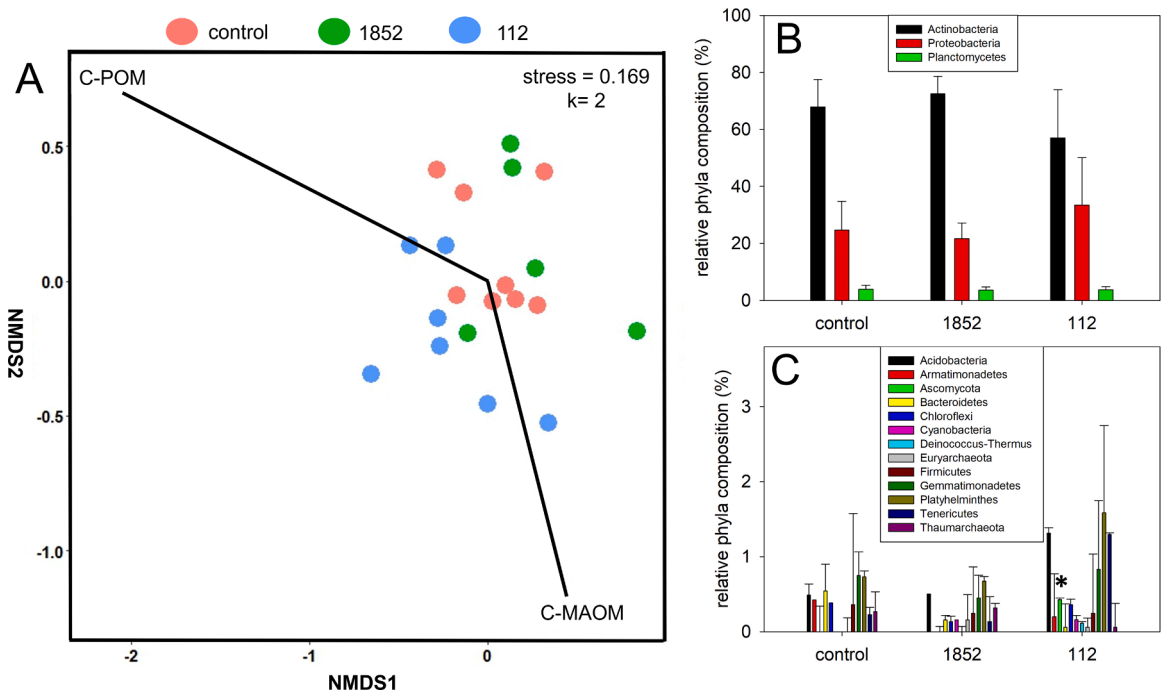

Fig. 4. Soil microbial composition determined via shotgun metagenomics in each pot of the plant growth experiment inoculated with fungi 112, 1852 or uninoculated (control). (A) Non-Metric Multi-Dimensional Scaling (NMDS) of soil microbial composition as relative percentage of all detected phyla and correlation with soil carbon and nitrogen parameters using the MANTEL test (only parameters with significant effects $p < 0.05$ shown). Results from Permutational Multivariate Analysis of Variance (PERMANOVA) to separate treatment effects area $p = 0.198$. (B) and (C) show soil microbial composition of common and rarer phyla (<3 % of total composition) respectively, as mean and standard deviation. Asterisks mark statistically significant differences determined by one-way ANOVA followed by Dunnett's post hoc test comparing fungi treatments to the controls ($p < 0.05$). C-POM, carbon associated with the particulate organic matter fraction; C-MAOM, carbon associated with the mineral-associated organic matter fraction.

Dunn test with Benjamini and Hochberg adjustment that did not show a p-value < 0.05 for control-112 ($p = 0.067$) and control-1852 ($p = 0.090$). We subsequently selected both treatments for detailed soil carbon fractionation analysis to assess whether statistically significant differences between the treatments can be detected between the three different soil carbon pools of different stability. Changes with other fungal treatments were not significantly different among biological pot replicates and were not further analysed. Aboveground plant biomass was not significantly affected by the two fungal isolates, yet one of the other 15 fungal isolates increased plant biomass (S1 Fig.).

3.2. Soil carbon fractionation assay on sub-set of samples

The rhizosphere soils of control and fungal isolates 112 and 1852 were subsequently fractionated into POM, AggOM and MAOM. The N associated with the POM fraction was the most sensitive parameter and increased by 93 % on average compared to the control in the samples treated with fungi 112 (Fig. 3B; $p = 0.002$), while the C content in the POM fraction was not different to the control (Fig. 3A). In isolate 1852, there was no change in POM-N and POM-C compared to the control.

The C and N contents associated with the AggOM fraction in the 112-isolate treatment increased significantly by 21 % (Fig. 3C; $p = 0.016$) and 34 % (Fig. 3D; $p = 0.010$; S3 Table), respectively, but no differences in treatment 1852 were detected.

Both isolates significantly increased the C and N content associated with the MAOM fraction by 8–10 % (Fig. 3E; 112: $p = 0.021$; 1852: $p = 0.005$) and 14–16 % (Fig. 3F; 112: $p = 0.0005$; 1852: $p = 0.0001$), respectively. The N content associated with the POM fraction correlated positively with both C in AggOM and MAOM fractions (S2 Fig.; AggOM and MAOM: $p = 0.02$).

We found a significant increase in recalcitrant C in the AggOM fraction in soil treated with isolate 1852 by 35 % ($p = 0.022$) but no increase in treatment 112 (Fig. 3D; grey bars). No significant change in recalcitrant C was detected within the MAOM fraction in either of the fungi treatments.

3.3. Microbial composition based on whole metagenome sequencing - links with C fractions

NMDS plot did not demonstrate a statistically significant shift in microbial composition due to fungi addition (Fig. 4A). The soil samples in the pots that were inoculated with 112 separated from the remaining samples through a shift in both NMDS 1 and 2, however, using pairwise PERMANOVA, we could not confirm a statistically significant shift in the microbial composition as a result of microbial inoculation (treatment effect p-value = 0.198). While there is also no visible change in the major microbial groups (Fig. 4B), there is an increase in microbial diversity as shown by Shannon diversity indices that significantly increased because of inoculation from 0.86 ± 0.07 in the control to 0.97 ± 0.07 in treatment 112 (ANOVA treatment effect: 0.004, Dunnett's test: 0.038) (1852: no significant change; 0.78 ± 0.11). The results indicate that the addition of fungi 112 stimulated a more diverse microbial composition.

Treatment 112 showed a significantly higher abundance of the phylum Ascomycota ($p = 0.005$), which is the phylum of both added fungi 112 and 1852 (Fig. 4C). This phylum was detected in six of the eight replicates of treatment 112, averaging ~0.5 % of the sequences detected in the soil (0 % in the control soil). All sequences were attributed to the family of the added fungi (Trichocomaceae) suggesting that at least in 6 of the 8 replicates the fungi successfully multiplied in the root and soil environment in detectable quantities. However, Ascomycota were not detected in any samples of the 1852 treatments (nor in the control) (Fig. 4C) suggesting that 1852 was not present in large enough quantities in the soil environment to be detected via non-targeted shotgun metagenomics as used here. There is a higher standard deviation in Actinobacteria and Proteobacteria in treatment 112 compared to

the other two treatments (Fig. 4B)

Statistically linking fractionation and microbial data (via Mantel test) revealed that the C content associated with the POM fraction changed significantly with microbial composition ($p = 0.014$; Fig. 4A). This change, however, was independent of microbial inoculation; samples treated with fungi 112 cluster in the left bottom corner in Fig. 4A, while the vector of C associated with POM (C-POM) is directed towards the left top corner, i.e., the changes are perpendicular to each other suggesting there is no correlation. Linear regression analyses of the main microbial groups demonstrate that the percentage of Proteobacteria positively correlates with POM content (Fig. 5), but not AggOM or MAOM contents (S3 Fig.). The percentage of Proteobacteria and the content of C-POM did not change statistically significant because of inoculation (Fig. 4B, Fig. 3A), but the Proteobacteria content dictates changes in microbial composition as determined by NMDS. In addition to C associated with POM, the C associated with the MAOM fraction correlated marginally statistically significant ($p = 0.044$) with the microbial composition (Fig. 4A).

3.4. *Predicted functional genes from whole metagenome sequencing - links with C fractions*

The (predicted) relative gene abundance based on COG categories were comparable in all treatments; there were no significant differences (S4 Fig.). Subsequent clustering of the functional genes in the soil samples based on KEGG Orthology (Fig. 6) also did not show a significant effect of fungal inoculation (PERMANOVA p-value: 0.212). However, similar to the microbial composition, using the Mantel test the C content within MAOM and POM significantly correlates with relative

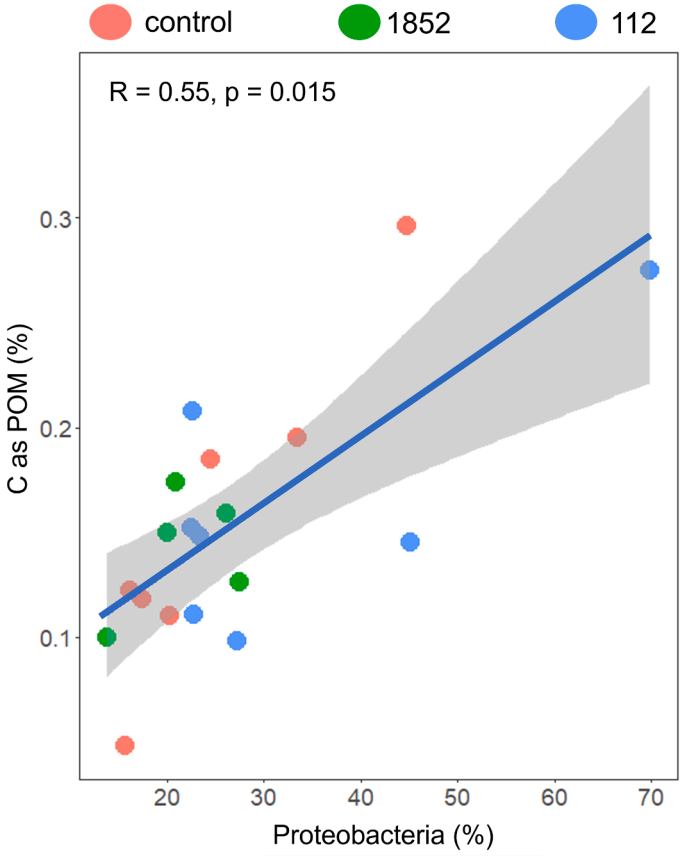

Fig. 5. Correlation of relative abundance of Proteobacteria (determined via shotgun metagenomics) and carbon content in soil (%) present as particulate organic matter (POM). Spearman rank correlation coefficient and significance value shown in top left corner.

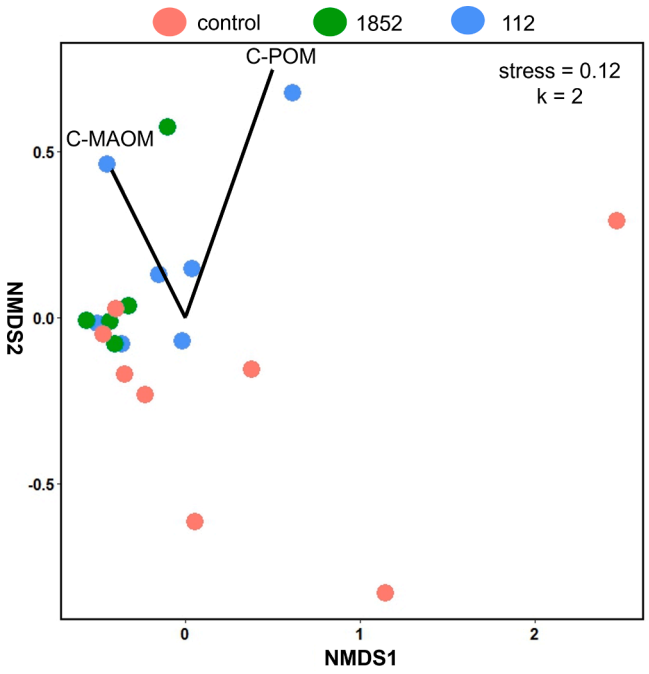

Fig. 6. Non-Metric Multi-Dimensional Scaling (NMDS) of (predicted) functional gene abundance determined via shotgun metagenomics. Data based on KEGG Orthology (KO) of DNA extracted from soil samples of the plant growth trial inoculated with fungi 112, 1852 or uninoculated (control). Results from correlation with soil carbon and nitrogen parameters using the MANTEL test (only parameters with significant effects $p < 0.05$ shown) and Permutational Multivariate Analysis of Variance (PERMANOVA) to separate treatment effects are $p = 0.212$. C-POM and C-MAOM = carbon associated with the particulate organic matter fraction, and mineral-associated organic matter fractions, respectively.

functional gene abundance (based on KEGG Orthology) (p-values 0.034 and 0.017, respectively).

The vector indicates the change of MAOM are oriented towards the opposite direction of where the control samples are clustered, suggesting carbon fractions, functional gene changes and inoculations are linked. The C content associated with the POM fraction also changed significantly, however independently of microbial inoculation treatments (Fig. 6A).

Various predicted functional genes (KEGG Orthology) showed significant differences among the treatments, mostly in treatment 1852 (S5 Table). The functional genes that were significantly different among the treatments are involved in various cell functions according to the COG categories, including co-enzyme transport and metabolism, inorganic ion transport and translation, ribosomal structure and biogenesis. Only the relative abundance of glycerol-3-phosphate dehydrogenase was significantly different in both microbial treatments compared to the control (SI Table 5).

3.5. *Phospholipid fatty acid (PLFA) - links with C fractions*

The PLFA content as indicator of total microbial biomass in soil was significantly higher ($p < 0.001$) in treatment 112 (4.26 μg $g^{-1} \pm 0.42$) compared to the control (3.40 μg $g^{-1} \pm 0.40$). This was attributed to a significant increase in gram positive bacteria, gram negative bacteria and fungi (Fig. 7). The fungal to bacterial ratio was not altered by either of the inoculants.

The fungal markers and total microbial biomass are significantly correlated with C in AggOM, C in MAOM and N associated with the POM fraction (S5 Fig.).

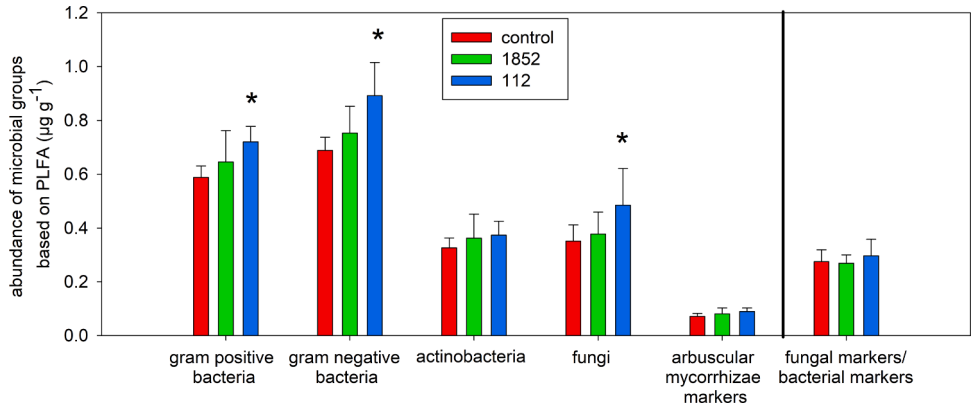

Fig. 7. Main microbial groups based on phospholipid fatty acid (PLFA) analysis as absolute content in soil (µg g^{-1}) or as ratio (fungal/bacterial markers: dimensionless). Total PLFA contents extracted for various groups as follow, control: 3.40 µg g^{-1} ± 0.42, 1852: 3.63 µg g^{-1} ±0.53 and 112: 4.26 µg g^{-1}±0.40. More details with results of statistics in S6 Table. Asterisks mark statistically significant differences determined by one-way ANOVA followed by Dunnett's post hoc test comparing fungi treatments to the controls ($p < 0.05$).

4. Discussion

4.1. Under controlled conditions some fungi inoculants can increase SOC in the rhizosphere

In this study we detected 15 % (extra) accumulation of SOC after wheat seed inoculation with two out of 17 fungi (Fig. 2). In comparable studies (but in soils with different starting SOC contents) using endophytes on *Phaseolus vulgaris, Trifolium subterraneum* and *Triticum aestivum*, the SOC contents increased significantly by 7 % in two out of eight endophytes (Alves et al., 2021), by 4–17 % (average by 10 %) in 20 out of 24 endophytes (Mukasa Mugerwa and McGee, 2017) and by 7–10 % in three out of twelve endophytes (Stuart et al., 2024), respectively. Together, our and literature results demonstrate that some endophytes can have potential to increase the SOC content in the area surrounding the roots by up to ~15 % in one growing season under controlled conditions in pot trials.

For detecting differences in SOC stocks due to microbial management, performing pot trials helps assess the potential of such treatments that they might show in the field, yet actual field trials, including temporal measurements are required. Some of this potential of fungal inoculants has now been also realised in field trials (Singh et al., 2025). While the rhizosphere is an effective way of detecting small differences in short-term experiments in both pot and field, as shown in our and other studies, this does not accurately represent the SOC content in the whole soil and because the effect is spatially explicit, such experiments should not be used to extrapolate the effect across an agricultural field. Measurements in-between planted rows are needed to assess the magnitude of changes taking spatial soil C dynamics into account. Finally, whether and how this intrinsic potential of microbial inoculants to increase SOC can express in different soils and climates and using other plant species also requires testing.

4.2. SOC increase could not be clearly associated with direct or indirect inoculant effect

The observed change in SOC could either be a result of more net plant C deposition (input minus loss) into soil or reduced loss of pre-existing soil C. The extra C was not attributed to the labile and recently plant derived POM fraction and, therefore, is not simply a result of additional plant root biomass (which would be present in the POM fraction). Consequently, the C accumulated in soil likely originated from the low-molecular weight (dissolved) plant C pathway (exudation). Plant exudates either directly accumulate in soil or are first converted into microbial biomass through anabolism, which subsequently deposits in soil as microbial necromass (Buckeridge et al., 2022; Camenzind et al., 2023; Sokol et al., 2019; Sokol and Bradford, 2019). Research suggests that both processes likely contribute equally to SOC formation under natural conditions (Angst et al., 2021).

Here, we found evidence for more microbial biomass of fungi and bacteria and higher diversity in the soil surrounding the roots after endophyte inoculation and increased chemical recalcitrance within the AggOM fraction (Fig. 3C), which indicates compounds more resistant to breakdown, as previously shown for melanin and other microbial cell envelopes (Fernandez et al., 2016; Throckmorton et al., 2015). While the increased microbial biomass may have contributed directly to increased soil C, the extra SOC more likely results from changes in microbial processes that boosted growth of non-endophytic fungi and bacteria (indirect changes) (Stuart et al., 2024).

Of the functional genes with significant changes in relative abundance, glycerol-3-phosphate dehydrogenase is involved in carbon metabolism, such as processing of glycerol that can be used by many microorganisms as source of carbon and energy (da Silva et al., 2009; Klein et al., 2017; Yeh et al., 2008), but is also involved in phosphorous acquisition (Gao et al., 2024) that in turn affects soil carbon cycling through potential destabilisation of soil carbon protection (Ding et al., 2021). The role of glycerol-3-phosphate dehydrogenase in soil carbon cycling and stabilisation could be an interesting future research topic and could be a target for improving SOC accrual. The other functional genes identified in this analysis are mostly involved in general cell functions and their changes in relative abundance likely resulting from shifts in microbial compositions but not necessary resulting in changes in carbon metabolism.

4.3. Rapid soil carbon fractionation assay as tool for evaluating effects of microbial management on long-term SOC storage and to generate big data for model predictions

The effect of fungi inoculants and other agricultural management changes on carbon of different stability need to be investigated in large field trials under different soil and environmental conditions to ensure predictable effects. For long-term carbon sequestration, it is essential to use SOC fractionation assays that can ultimately help separate out the labile POM fraction that both sharply increases, e.g., when crop residues are returned to the field, and also quickly decreases due to microbial decomposition. Separating out POM takes out a background SOC signal and increases the likelihood of detecting changes to long-term SOC accrual. However, common approaches to measure SOC pools across field trials are typically labour-intensive and slow, bringing challenges when analysing large number of soil samples. Rapid soil carbon fractionation assays, such as the one presented here, could help overcome such limitations.

We show that in soils with low starting POM content, POM-N shifts from the baseline after microbial inoculation could potentially be an early indicator for future SOC accrual. This increase in N is likely a result of microbial biomass within the POM fraction. Among the three soil fractions, the POM fraction had the highest C/N ratio by far (control treatment C/N ratio 52) as also reported in other studies (Blume et al., 2016). The C/N ratio of fungi and bacteria is much lower (10 and 4 on average, respectively (Liang et al., 2019) and therefore, an increase in microbial abundance is detected readily in the form of extra N in the POM fraction. N-POM could indeed be linked with higher fungal abundance (S5 Fig.). It further correlated significantly with C in the AggOM and MAOM fractions where decomposed microbial fragments would subsequently deposit (S2 Fig.). This relationship will have to be tested in larger studies in the field but could be a simple measure that indicates SOC changes and does not require any additional measurements.

Ultimately, there is an urgent need to better link soil carbon of different stability with microbial indicators. As we show here, the POM content was linked with the relative abundance of Proteobacteria (Fig. 5), which are known to increase as a response to easily available C in soil (Eilers et al., 2010; Fierer et al., 2007) supporting the labile nature of POM as determined in this assay (S3 Fig.). It highlights that the more stable and labile C stocks are likely influenced by different microbial indicators and large sets of soil samples could be measured using the high-throughput analyses pipeline described in this manuscript to further link soil C of different stability with microbial indicators. This could allow to generate large data sets that can be used to calibrate models, such as the Millenial or MEMS (Abramoff et al., 2022; Zhang et al., 2021), to better predict changes of SOC on small- and large-scale and enable future predictions.

5. Conclusions

Under specific soil and environmental conditions in a pot trial, an endophyte inoculant increased stable SOC through increased microbial abundance and diversity. A second endophyte also increased stable SOC content, which is likely linked to changes in functional genes. We further show that the more labile SOC fraction, POM, was related to different factors (relative abundance of Proteobacteria) than the more stable, AggOM and MAOM, fractions (microbial biomass). More detailed investigations of the underlying mechanisms behind these links could bring insight into the types of microbial interactions driving soil C fractions. High throughput soil C fractionation and metagenomics assays, such as the ones presented here, could help establish early microbial indicators for changes in soil C content of different stability by evaluating large scale, field studies and ultimately be used to calibrate soil C models.

CRediT authorship contribution statement

Yolima Carrillo: Writing – review & editing, Resources, Investigation. **Justin Borevitz:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Wolfram Buss:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Scott Ferguson:** Writing – review & editing, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Justin Borevitz reports financial support was provided by Australian National University. Justin Borevitz reports financial support and equipment, drugs, or supplies were provided by SoilCQuest. Justin Borevitz reports a relationship with SoilCQuest that includes: board membership. JB received test fungal inoculants and parts of the funding for this work from SoilCQuest 2031 in 2020. JB became a member of the research committee of SoilCQuest in late 2023, after the experiments for this manuscript were conducted and a draft submitted (bioarchive Dec 2021) for publication. SoilCQuest is a non-for-profit organisation focused on agronomy and soil health that has shares in Loam Bio, a company commercialising fungal inoculants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2025.109798.

Data availability

Data will be made available on request.

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