Title: A diversity of fungal pathways contribute to improved soil carbon stability and storage

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Abstract

While various fungi could facilitate soil C storage and climate change mitigation via carbon (C) cycling, standard soil C tests that measure only bulk soil C cannot disentangle mechanisms underpinning fungal influences and so far research has largely focused on mycorrhizal fungi. Here, we assessed the soil C storage potential of 12 non-mycorrhizal fungi, selected from a wide pool based on traits potentially linked to soil C accrual. We grew wheat plants inoculated with individual isolates in chambers designed to differentiate plant- and soil-derived C using stable isotope analysis. After harvest, we conducted long-term soil incubations and high throughput fractionation to determine fungal impacts on soil C pools. While only some isolates resulted in significant total soil C increases, most significantly improved soil C stability by increasing the stable pools of soil C, providing the first direct experimental evidence that inoculation with specific fungi can improve soil C storage by

stabilising existing C. These increases were positively associated with fungal and plant growth characteristics, indicating direct and indirect mechanisms for fungal impacts on soil C storage. Our study highlights the need for more research on the roles of non-mycorrhizal fungi in C cycling and for higher resolution methods to understand fungal impacts on soil C storage, as these fungi hold promise for soil C sequestration strategies.

Introduction

Despite soils having the capacity to sequester large amounts of atmospheric CO_2 and mitigate catastrophic climate change impacts, the full potential of soil carbon (C) sequestration is yet to be realised (Field & Raupach 2004, Scharlemann et al. 2014, Schlesinger 1990). Moreover, rather than being protected, soils are becoming increasingly degraded globally due to intensive agricultural practices that disturb soil structure - a situation that may worsen as decomposition potentially accelerates with future global temperature increases (Hannula & Morriën 2022, Lal 2018). While soil C sequestration is becoming more broadly recognised as an important climate change strategy, its successful implementation first requires a comprehensive understanding of processes underpinning soil C storage (Dynarski et al. 2020, Smith et al. 2019, Unger & Emmer 2018). Scientific knowledge of soil C storage has improved substantially in recent years, with it now understood to result from the balance of dynamic and complex processes determining C inputs and outputs. In contrast, previous hypotheses on soil C storage posited, for example, that it results primarily from inherently stable C inputs, or alternatively from repression of microbial degradation of C (Dignac et al. 2005, Dynarski et al. 2020, Haddix et al. 2016, Heim & Schmidt 2007, Jackson et al. 2017, Klotzbücher et al. 2011, Marschner et al. 2008). Furthermore, soil C storage is currently understood to be largely microbially-driven (Dynarski et al. 2020, Kallenbach et al. 2016, Prescott 2010). However, the soil microbial community is diverse, complex, and largely unexplored; hence, known as a "black box" (Mishra et al. 2022, Tiedje et al. 1999). Within the black box, fungi are considered particularly important for soil C storage as they directly and indirectly affect both C inputs and outputs, via multiple mechanisms occurring simultaneously (Fig. 1; Hannula & Morriën 2022, Kallenbach et al. 2016, Liang et al. 2019, Starke et al. 2021). Thus, the overall impact of a particular fungus on soil C storage difficult to predict, let alone the various fungal species present within a soil community.

Fungi may impact not only the quantity and quality of soil C inputs but also the stability of different C pools, influencing outputs. In general, fungi can impact soil C storage as follows. Fungi utilise and transform soil- and plant-derived C, forming microbial-derived organic matter, which can be sorbed onto mineral surfaces and thereby protected. Their impacts on soil structure and spatial heterogeneity, including promoting aggregate formation, further protects C (Berg & McClaugherty 2013, Dynarski et al. 2020, Kleber et al. 2011, Lützow et al. 2006, Schmidt et al. 2011). They may also stabilise soil C due to composition of their cell walls, which generally contain more complex compounds compared to bacteria, although the significance of some cell wall compounds for soil C storage may be overstated (Anthony et al. 2020, Berthelot et al. 2017, Cheeke et al. 2021, Clemmensen et al. 2015, Fernandez & Kennedy 2018, Fernandez & Koide 2014, Hannula et al. 2019, Throckmorton et al. 2012, Wang et al. 2017). The abilities of some fungi to alter plant growth, protect plants from pathogen attack, or influence the soil microbial community, influencing plant- or microbial-derived C in soil, are some indirect mechanisms by which fungi may influence soil C storage (Clocchiatti et al. 2020, Hannula & Morriën 2022, Rai & Agarkar 2016, Stuart et al. 2022).

These potential impacts of fungi on soil C storage are further complicated by fungal diversity, which occurs at the inter-genus, inter-species and even down to the sub-species level (Andrade et al. 2016, Hiscox et al. 2015, Johnson et al. 2012, Juan-Ovejero et al. 2020, Plett et al. 2021). In plant-soil ecosystems, fungi mainly exist either as free-living saprotrophs or as plant-associated fungi, including mycorrhizal, endophytic, and parasitic fungi (Rai & Agarkar 2016). Much of the research on fungal impacts on soil C has focused on mycorrhizal fungi, particularly arbuscular mycorrhizal fungi and ectomycorrhizal fungi due to their dominance in their respective habitats (Jackson et al. 2017, Smith & Read 2008). These fungi funnel plant C belowground and, in some cases, partially decompose organic matter, prime or suppress microbial respiration, or have cell walls containing melanin, which is considered to

be highly stable and has been associated with decreased hyphal decomposability and increased soil C content (Anthony et al. 2020, Averill & Hawkes 2016, Cheeke et al. 2021, Fernandez & Kennedy 2018, Fernandez & Koide 2013, Frey 2019, Gadgil & Gadgil 1971, Zak et al. 2019, Zhu & Miller 2003). In contrast, despite the large diversity amongst fungi in plant-soil ecosystems, influences of non-mycorrhizal fungi on soil C storage have not been studied in as great detail compared to mycorrhizal fungi. Some endophytic fungi may be important for soil C storage, due to their ability to produce melanin and promote plant growth (Berthelot et al. 2017, He et al. 2019, Mandyam & Jumpponen 2005, Rai & Agarkar 2016). However, there are conflicting reports regarding their lifestyles, benefits or harms imposed on host plants, enzymatic and nutrient acquisition ability, or even whether they produce extraradical mycelium, suggesting there may be wide functional variation or plasticity within this fungal group (Addy et al. 2005, Mukasa Mugerwa & McGee 2017, Rai & Agarkar 2016). Saprotrophic fungi are often assumed to predominantly influence soil C output, as they decompose soil organic matter due to being outcompeted by mycorrhizal fungi for plant C exudates. However, decomposition can increase the availability of C to be sorbed onto mineral surfaces, thereby fostering soil C stability (Frąc et al. 2018, Hannula & Morriën 2022, Lehmann & Rillig 2015). Pathogenic fungi are poor competitors for soil organic matter but may play major roles in soil C cycling by degrading living organic matter, making it available for saprotrophs (Thormann 2006). To better understand fungal impacts on soil C storage, particularly soil C stability, more focus is needed on fungal types other than mycorrhizal fungi.

To disentangle the various mechanisms underpinning fungal impacts on soil C storage, multiple, complementary methodologies are needed. Within soil, C is present as a continuum of materials ranging in stability (propensity to decomposition) and origin, and is conceptualised as different theoretical pools (Amundson 2001, Dynarski et al. 2020).

However, most studies that evaluate soil C tend to measure only bulk soil C, missing temporal and spatial nuances of C flowing through these pools. While fractionation methods may not exactly reflect natural soil C turnover and stability, these analyses that assess different C pools, via physical separation methods based on size or density, have offered more resolution in understanding soil C responses (Castanha et al. 2008, Kögel-Knabner et al. 2008, Poeplau et al. 2018). Greater still resolution can be achieved via long-term soil incubations, which assess natural soil C turnover or mean residence time (MRT) and allow for decomposition rates of separate C pools to be measured over time (Carrillo et al. 2011, Chenu et al. 2015). Combining these approaches with isotopic separation of plant- versus soil-derived C could increase resolution and explanatory power even further.

Here, we aimed to assess the effects of inoculation of soil with fungi other than mycorrhizal fungi on soil C storage (e.g. changes in inputs and stability), and to investigate direct and indirect mechanisms underpinning these effects (Fig. 1). We inoculated spring wheat (*Triticum aestivum*), an important cereal crop, with one of 12 fungi originally isolated from plant roots and screened for traits that may support soil C storage such as capabilities to capture and solubilise nutrients from the soil to support plant growth. We chose a crop plant as our study system as croplands have large but untapped potential in soil C sequestration (Amelung et al. 2020). We grew the plants for four months in ^{13}C-depleted CO_2 growth chambers to homogeneously label the plants during the growth cycle. Following harvest, we incubated root-free soil to assess the size and turnover of different C pools, and evaluated the contribution of soil and plant C to these pools using isotopic analysis. These C pool measures were accompanied by high throughput size and density fractionation analyses (hereafter we refer to the pools measured via fractionation analysis as “fractions”, as opposed to “pools” measured via soil incubations) to more extensively understand fungal impacts on C turnover and stability. We then measured characteristics of the fungi and of the plants and microbial

community to explore the direct and indirect mechanisms behind these impacts. We hypothesised that, with some variation, these fungi improve soil C storage primarily by stabilising soil C (decreasing outputs as opposed to increasing C inputs), and that fungi-driven increases in soil C would therefore be positively associated with stable pools or fractions of C. We also expected that there would be fungal, plant and microbial community variables associated with increased soil C stability, alluding to both direct and indirect mechanisms.

Materials and methods

See Supplementary Methods for further details.

Biological material and pot preparation

Pure cultures of 12 fungal isolates, including endophytic, saprotrophic and parasitic fungi, were originally obtained from plant roots and screened for traits that may support soil C storage by Loam Bio Pty Ltd (Orange, New South Wales, Australia). Surface-sterilised and moistened wheat seeds (*Triticum aestivum*) were incubated at room temperature for 48 h.

For "planted" replicates, three agar squares from actively growing fungal cultures were placed near three seeds in plastic pots containing 2 mm sieved, non-sterile, clay loam soil (4.3% C; Table S1). Uninoculated planted pots ("absent/control") received three agar squares from uninoculated plates. Each agar square contained approximately 1.3 mg C. Smaller pots for "unplanted" control pots (see below) were set up three days later using two agar squares. After 10 days of growth, seedlings were thinned to one per pot.

Experimental design and maintenance

The experimental setup consisted of seven planted replicates inoculated with one of the 12 fungal isolates, and six replicates of uninoculated planted pots, distributed among six growth chambers. Pots were regularly and uniformly watered with tap water. Four replicates of unplanted pots containing only fungal inoculum were included as controls, adding to 142 pots in total. Unplanted pots were kept under the same conditions as the planted pots for the duration of the experiment.

The CO_2-controlled growth chambers were modified using the approach by Cheng and Dijkstra (2007) to achieve continuous ^{13}C-labeling of plant tissues. Chambers were adjusted

to a 16 h/8 h photoperiod, 22°C/17°C, 60% relative humidity, and 500 µmol m^{-2} s^{-1} light intensity. The chamber atmosphere was checked weekly to confirm that the atmospheric CO_2 was sufficiently depleted in ^{13}C.

Harvest and plant biomass measurement

Once plants had senesced and the grain had ripened, at 18th weeks of growth, wheat spikes and shoots were cut off, dried at 70°C and weighed. The remaining soil was preserved in the pots by freezing at -20°C immediately after shoots were cut to stop all decomposer activity to retain the C status generated by the treatment until ready for subsampling and processing. Briefly, soil for fractionation analysis was collected from near the root crown and dried at 40°C. A third of the main root system was collected, washed, frozen at -20°C for root morphology measurement, and later dried at 40°C and weighed. After homogenisation of the remaining soil, soil for phospholipid fatty acid (PLFA) analysis was collected and frozen at -20°C. Soil moisture content was measured on soil dried at 105°C. For soil incubations, a subsample was dried at 40°C and sieved at 2 mm. Of this, a further subsample for isotope analysis was dried at 105°C. The root/soil ratio outside the main root system was calculated based on the remaining soil and roots after all subsampling, and used to estimate the root mass in the soil subsamples. Total root mass was determined by combining the weighed root mass to the estimated soil subsamples root mass.

Plant and soil isotope and chemical analysis

To determine the contribution of soil- versus plant-derived C to total C, isotopic compositions and C/N content of ground shoots and soil were assessed using an elemental analyser interfaced to a continuous flow isotope ratio mass spectrometer (UC Davis Stable Isotope Facility, Davis, California, USA). The proportion of original soil C present in the soil of each pot after plant growth was calculated via isotopic partitioning.

Soil incubations

To evaluate the impact of fungal isolates on size and degree of stability (i.e. susceptibility to decomposition) of soil C pools, we assessed microbial respiration during 135-day laboratory incubations under standard temperature and moisture conditions. Kinetic parameters derived from mid- to long-term soil incubation data are more sensitive indicators of soil C responses than bulk soil C measurements; thus, soil incubations can be used to assess changes in the stability of C pools resulting from previous exposure to experimental treatments (Carney et al. 2007, Carrillo et al. 2011, Jian et al. 2020, Langley et al. 2009, Taneva & Gonzalez-Meler 2008). Measured CO_2 production rates were fitted to a two pool exponential decay model in which two pools of C, one labile and one intermediate, decay exponentially (Cheng & Dijkstra 2007, Wedin & Pastor 1993). To calculate the contribution of plant- and soil-derived C to respired CO_2 at each sampling point, isotopic partitioning was used. These fractions were applied to the measured CO_2 amounts in each jar to calculate plant- and soil-derived amounts. Based on these, we calculated total CO_2 released from plant- and soil-derived C during the full length of the incubation.

Soil fractionation analysis

Soil fractionation analysis was performed according to a method developed by Poeplau et al. (2017, 2018) and adapted by Buss et al. (2023, in review) involving high throughput physical fractionation into conceptually designed soil C fractions - mineral-associated organic matter (MAOM), aggregate carbon (AggC), and particulate organic matter (POM). Carbon and nitrogen (N) content of the fractions were measured via combustion analysis.

Root morphology

To evaluate root morphology, root subsamples were arranged with minimal overlap for digital scanning (Epson Expression 11000XL scanner, Epson, Macquarie Park, Australia). Images were analysed with WinRhizo Pro software 2015 (Regent Instruments Inc., Quebec City, Canada).

In vitro fungal assessment

To assess morphological and chemical properties of the fungal isolates, an *in vitro* plate assay was performed using 1/2 potato dextrose agar plates incubated in the dark at 23-25°C. Radial growth rate was calculated by measuring colony areas every two-to-three days using ImageJ (National Institutes of Health, Bethesda, Maryland, US; Schneider et al. 2012). Growth rate was calculated by subtracting the colony area from an earlier sampling point from that of the following sampling point. Hyphal density was calculated as the final fungal biomass per final colony area. C and N content were measured by Dumas combustion using a vario EL cube analyser (Elementar, Langenselbold, Germany).

Soil PLFA analysis

Microbial PLFAs in soils were extracted from 2 g of freeze-dried soil following the high throughput method developed and described by Buyer and Sasser (2012) and Joergensen (2022).

Data and statistical analysis

ANOVA of soil C properties and experimental variables was performed in R (v. 4.1.2; R Core Team 2021), followed by Dunnett's post-hoc test to determine which treatment groups were significantly different to the uninoculated control group or Tukey’s post-hoc test to determine significant differences between inoculated groups. Principal component analysis

(PCA) and redundancy analysis (RDA) were performed using the vegan package in R (Oksanen et al. 2020).

Curve fitting of CO_2 rate dynamics was done using the non-linear modeling platform in JMP 16.1.0 and the biexponential four-parameter decay model using all replicates of a treatment. We used nonlinear least square curve fitting to test if the parameters were significantly different between a fungal treatment and uninoculated control, using the nls function in R.

Results

1. Fungi increase soil C under wheat plants

We inoculated wheat plants (*Triticum aestivum*) with one of 12 fungi (non-mycorrhizal) isolated from plant roots. After four months of plant growth, there was a positive but varied effect of fungal inoculation on soil C content compared to the uninoculated control group ($p < 0.05$; Fig. 2, Table S2). This effect was not observed in soils that received the same fungi but were unplanted ($p = 0.22$; Fig. 2). We found significant isolate-specific increases in soil C content of the planted treatments under inoculation with Chaetosphaeriaceae sp., Lentitheciaceae sp. 3, and Morosphaeriaceae sp., relative to the uninoculated control, of 9.4% (± 1.12, $p < 0.01$), 7.5 (± 0.50, $p < 0.05$), and 7.8 (± 0.76, $p < 0.05$), respectively (Fig. 2; Table S2). Nitrogen levels were generally higher in the soils of the inoculated and planted treatments compared to the uninoculated control ($p < 0.05$; Table S2).

2. Fungi-dependent increases in soil %C are associated with changes in soil C properties

To understand the underlying mechanisms of the fungal isolate-dependent increases in soil C content and potential shifts in the stability of the resulting soil C, we measured an array of soil C properties. We performed C isotope analysis, soil incubations, and soil C fractionation analysis to gain insight into the origin and stability of soil C. While planting reduced total soil C, as expected as C inputs into soil stimulate decomposition (rhizosphere priming), the amount of reduction was dependent on fungal treatments (Fig. 3, Table S2). Based on isotopic partitioning of C in soils from planted pots, at the time of harvest an average of 3.8% (±0.2) of C in soil was plant-derived, as opposed to soil-derived (Fig. 3, Table S2). This amount varied across treatments and resulted in significant changes in plant-derived C. One of the fungal treatments whereby total soil C significantly increased (Chaetosphaeriaceae sp.)

exhibited higher amounts of plant-derived C compared to the controls at a level that was marginal in its non-significance ($p < 0.1$; Fig. 3a, Table S2). However, overall, the increases in total soil C corresponded more closely with increases in soil-derived C ($R = 0.93$, $p < 0.01$), than with plant-derived C ($R = 0.02$, $p = 0.83$). All three fungal treatments resulting in significant increases in total soil C showed increases in soil-derived C but these were not statistically significant (Fig. 3b, Table S2).

Soil incubations after wheat growth revealed fungal effects on fractions of the total, soil-derived C and plant-derived C that were available for decomposition after plant harvest (fraction of what was present at harvest that was respired over the full incubation). Significantly lower proportions of the total and soil-derived C fractions were respired under all fungal treatments compared to the controls ($p < 0.001$), while the plant-derived respired C fraction was significantly lower only under inoculation with Mollisiaceae sp. 1 ($p < 0.05$; Table S2). There was also a significant fungal effect on the estimated amount of total C remaining after incubations ($p < 0.05$; Table S2). The dynamics of total C decomposition (decay models derived from incubations) of soil after wheat growth showed significant fungal effects compared to the uninoculated controls under inoculation with Chaetosphaeriaceae sp., Lentitheciaceae sp. 2, Lentitheciaceae sp. 3, Mollisiaceae sp. 1, Mollisiaceae sp. 2, and Morosphaeriaceae sp. (Table S3, Fig. S1). Soil-derived C decomposition dynamics were also significantly different to the controls under the same fungal treatments as well as Leptodontidiaceae sp. (Table S3, Fig. S1).

The soil C pool sizes estimated from these decay models demonstrated significant effects of fungal inoculation on total ($p < 0.001$) and soil-derived resistant C ($p < 0.01$). Significantly higher resistant C, compared to controls, was observed under inoculation with specific isolates, including Chaetosphaeriaceae sp., Lentitheciaceae sp. 3, and Morosphaeriaceae sp.

for total resistant C, and Chaetosphaeriaceae sp. and Morosphaeriaceae sp. for soil-derived resistant C (Table S2). Statistical results for labile and intermediate C pools could not be acquired as they were calculated using treatment averages (Table S2). In terms of labile C, MRT of the total C was significantly lower under inoculation with Lentitheciaceae sp. 1 compared to the control, whereas MRT of the soil-derived C was significantly higher under inoculation with Periconiaceae sp. In terms of intermediate pool MRTs, controls and fungal treatments were not significantly different.

From fractionation analysis, %C and %N of the AggC fraction, i.e. the fraction of intermediate stability whereby C is protected in aggregates, were found to have significant fungal effects, with Chaetosphaeriaceae sp. ($p < 0.001$) and Periconiaceae sp. ($p < 0.05$) exhibiting significantly higher levels of both C and N, and Phaeosphaeriaceae sp. ($p < 0.05$) and Mollisiaceae sp. 1 ($p < 0.01$) exhibiting significantly higher levels of only N compared to the uninoculated controls (Table S2). Significant fungal effects were not observed in the MAOM and POM fractions (Table S2).

We performed PCA to identify soil C properties associated with fungi-driven increases in soil %C (Fig. 4). Most of the variance was explained by PC1 and 2 (62%). Greater soil %C was closely associated with soil-derived C at time of harvest and C remaining in soil after incubations. Soil %C was also related, to a slightly lesser extent, with total and soil-derived resistant C pools. These soil C properties (and soil %C) were positively associated with Chaetosphaeriaceae sp. The other fungal treatments that resulted in significant increases in soil C% (Morosphaeriaceae sp. and Lentitheciaceae sp. 3) were somewhat related with these soil C properties and also to %C in the POM fraction. %C of the AggC and MAOM fractions, considered to be more stable fractions of C, were not clearly associated with soil %C or the resistant C pools, nor with any fungal treatments. The uninoculated controls were separated

from these three fungal treatments and instead trended positively with total and soil-derived respired C.

3. Fungi-dependent increases in soil C and its stability are positively associated with plant growth

We assessed plant and microbial community variables, including plant biomass, shoot C/N content, root morphology, and total microbial community size and composition derived from PLFA analysis. Overall, while variation among fungal isolates was observed, no significant differences were observed between the inoculated and uninoculated plants for any of the plant or microbial community variables measured, although average spike mass of Chaetosphaeriaceae-inoculated plants was significantly higher than that of uninoculated plants (Table S4).

To identify plant and microbial community variables potentially involved in the fungal isolate-dependent changes in soil C properties, we performed RDA using plant and microbial community data and the soil C property data used in the PCA (Fig. 5). Variance explained by RDA1 and 2 was 14.1 and 5.7%, respectively. The cluster of soil C properties that were found to be closely associated with Chaetosphaeriaceae sp. in the PCA (e.g. soil-derived C, resistant C pools; Fig. 4) also trended positively with plant biomass and growth (spike and shoot mass, shoot C/N ratio, and root fork number). While PLFA-assessed soil microbial community variables were generally not associated with the fungal isolates with significant soil C increases, there were somewhat positive associations between percentage of arbuscular mycorrhizal fungi and Lentitheciaceae sp. 1, Lentitheciaceae sp. 2, and Phaeosphaeriaceae sp., as well as between percentage of gram positive bacteria and Lindgomycetaceae sp. The uninoculated controls and their associated soil C properties (i.e. respired C pools) were somewhat related to plant shoot N and root specific density.

4. Fungi-dependent increases in soil C and its stability are associated with denser fungal hyphae

Fungal isolates showed strong differentiation in the *in vitro*-assessed variables relating to growth and C/N content (statistically significant effects on all variables, $p < 0.001$; Table S4). Isolates with higher biomass values tended to have higher colony areas and growth rates. These included Lentitheciaceae sp. 2, Didymosphaeriaceae sp., Periconiaceae sp., Phaeosphaeriaceae sp., and Morosphaeriaceae sp. (Table S4). In contrast, Lindgomycetaceae sp. and Chaetosphaeriaceae sp. tended to have lower values for these variables.

We performed a separate RDA to identify fungal variables potentially involved in fungi-dependent soil %C and soil C stability increases, using *in vitro* fungal assessment data and the soil C property data (Fig. 6). Compared to the RDA using plant and microbial community data (Fig. 5), greater proportions of variance were explained in this RDA by RDA1 and 2 (22.7 and 9.5%, respectively). Fungal colony area and hyphal density were close to opposite in their direction, with Chaetosphaeriaceae sp. closely associated with hyphal density, and Lindgomycetaceae sp. associated with colony area. Similarly, fungal colony maximum growth time and rate (denoting slower and faster fungal growth, respectively) were in opposing directions. Along this axis, Lentitheciaceae sp. 1 and Phaeosphaeriaceae sp. trended positively with maximum growth time, and Lentitheciaceae sp. 3 and Morosphaeriaceae sp. were more associated with maximum fungal growth rate. Lentitheciaceae sp. 2 trended positively with hyphal C/N ratio and biomass.

Discussion

Discussions on soil C sequestration as a climate change strategy have largely focused on one side of the soil C storage system - increasing C inputs into soil. However, due to the dynamic nature of soil C storage, in order to promote soil C storage, reductions of soil C outputs must also be attained. In this study, we drew our attention to fungi that have potential in improving soil C storage but that are often overlooked in this area of research, using a high resolution, multifaceted approach combining isotopic labeling, soil incubations and soil fractionation analysis. Despite our finding that bulk soil C increased significantly under only three fungal treatments, in support of our hypothesis our incubations revealed significant increases in directly and functionally assessed soil C stability (i.e. increases in resistant pools and remaining C, decreases in respired C during incubation) under most of the fungal treatments, with the stabilised C being original soil C, not new inputs of plant-derived C. Thus, as well as contributing to evidence that fungi can lead to increased soil C content (e.g. Kallenbach et al. 2016), our study provides the first direct evidence for fungi-driven increases in soil C storage via greater C stability. Stabilisation of soil C is vital for facilitating soil C storage, as without stabilisation, C inputs would be susceptible to loss via decomposition (Lal 2018). Therefore when it comes to evaluating the potential of fungi to support soil C storage, our findings indicate that it is important to consider not only fungi-driven increases in soil C but also their impact on the stability of C within soil. The significant fungi-driven increases in stability we observed could potentially lead to even greater increases in soil C content over time, however experiments with longer timeframes are needed to test this idea. Our study supports the general agreement in this field that microbial transformations of soil C are as important, if not more important, than the characteristics of the inputs themselves for soil C storage (Dynarski et al. 2020, Hannula & Morriën 2022). Although we were not able to demonstrate survival of the fungal isolates, the observed fungal treatment effects support that there were fungi-driven

impacts on soil C storage. This is also further supported by fungal treatment effects seen in planted soils, as opposed to unplanted soils, indicating fungal-plant interactions.

From our fractionation analysis, the fractions considered to signify increased and longer-term stability - aggregate and MAOM fractions (Dynarski et al. 2020, Hemingway et al. 2019, Islam et al. 2022, Poeplau et al. 2017, 2018) - were not strongly associated with soil C content and its stability as determined via the soil incubations, nor were they as influential as the soil incubation pools on differences between fungal treatments. A potential explanation for our findings is that the experimental conditions may have been unsupportive of MAOM formation (e.g. the high C content of the unplanted soil may have meant that MAOM content was already at saturation level and new MAOM was not able to form). This would suggest that the increases in soil C observed in this study may not have been sustained long-term as new C inputs could not be transformed into MAOM, although this may not necessarily be the case as recent work has demonstrated that the MAOM fraction is likely diverse in its stability and turnover time and therefore may not be uniformly affected by experimental conditions (Sokol et al. 2022). Other potential explanations are that the MAOM fraction could possibly take longer than the experimental timeframe to change substantially, or that soil fractionation analyses do not entirely accurately reflect natural soil distribution and stability. Further studies utilising the combined approach of soil incubations and soil fractionation analysis, such as studies using soil with lower C content or studies over a longer time period, may shed light on how findings from the two methods can be compared. However, our findings call for caution in equating operationally defined MAOM pools and their size with C stability.

Our study provides a theoretical framework in which fungi may influence soil C storage via indirect and direct mechanisms. One of the major findings of our study is that of the three fungal isolates resulting in significant increases in soil C, these were accompanied by

increases in plant-derived C only under inoculation with Chaetosphaeriaceae sp. While we expected that there would be some variation in the fungal impacts on soil C storage due to the diversity amongst the fungi included in this study, this finding suggests that there may be other potential mechanisms by which fungi increase soil C storage (i.e. not just transformations that stabilise C). The increase in plant-derived C with Chaetosphaeriaceae sp. may have been related to the increases in plant inputs related to the shifts in plant variables of Chaetosphaeriaceae-inoculated plants as opposed to Morosphaeriaceae sp. and Lentitheciaceae sp. 3 (spike mass, shoot biomass, and shoot C/N ratio). Plant growth promotion is a well known characteristic of some fungi, particularly the promotion of shoot growth as opposed to root growth, potentially as the plants invest less into root growth as they become more dependent on fungi for soil nutrient acquisition (Hossain et al. 2017). Thus, increased plant biomass may potentially be an indirect mechanism by which some fungi increase C inputs into soil. Fungal-derived C could also theoretically contribute to the plant-derived C fraction, if fungi take up or consume plant-derived C. The findings of our study also highlight fungal growth traits that may constitute a direct mechanism by which fungi influence soil C storage. More precisely, we found in the fungal assay that hyphal density and radial growth rates may be relevant traits for soil C storage, with Chaetosphaeriaceae sp. (characterised by denser hyphae) and Morosphaeriaceae sp. and Lentitheciaceae sp. 3 (characterised by faster growth) all significantly increasing soil C. This supports previous assertions that fungal trait expression can be relevant to soil C stability, and also hints that fungal trait expression may help determine the different ways in which these fungi influence soil C storage (Camenzind et al. 2020, Fernandez et al. 2019, Fernandez & Koide 2013, Jackson et al. 2017, Lehmann et al. 2020, Schmidt et al. 2011, Zanne et al. 2020). Although it should be acknowledged that fungal growth is highly dependent on external conditions such as nutrient availability, and therefore fungal growth under *in vitro*

conditions may not reflect that within soil (Bekker et al. 2006, Gadd et al. 2001, Parrent & Vilgalys 2007, Suberkropp 2011), our study provides clear evidence of linkages between fungal traits and soil C storage.

Our study addresses key knowledge gaps in the ways fungi affect soil C storage. We have explicitly demonstrated that inoculation with non-mycorrhizal fungi can improve soil C content and, moreover, soil C stability. These improvements are largely driven by reductions in C outputs by increasing stable C pools and resistance of existing soil C to decomposition, but mechanisms behind these improvements may depend on fungal identity and growth characteristics. The improvements to soil C storage may also involve the effects of fungal inoculation on host plant growth and C inputs. More research is needed to further understand the direct and indirect mechanisms by which these fungi impact soil C storage, including experiments with longer timeframes and further comparisons of the methods used in this study to assess soil C pools. This study and continued work will advance knowledge of these mechanisms and identify fungi that may improve soil C storage, which will aid the implementation of soil C sequestration strategies.

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Figures

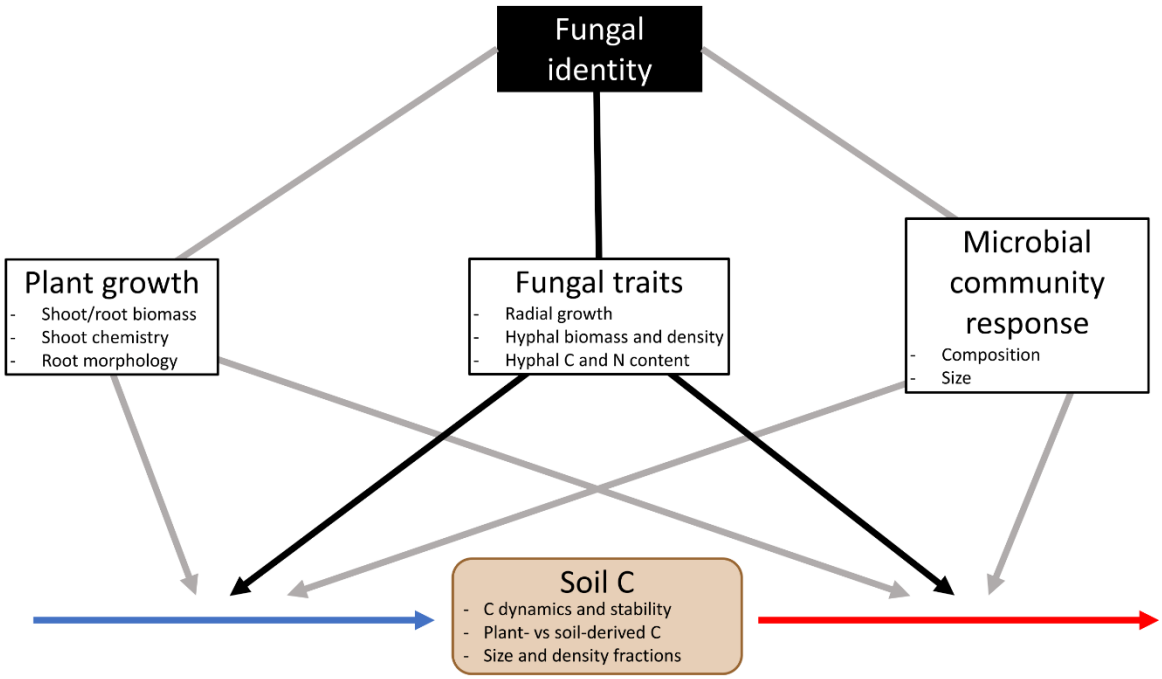

Figure 1. Examples of the potential pathways of influence of fungi on soil C storage and the specific drivers (white boxes) and response variables (tan box) assessed in this study. Blue arrows indicate soil C inputs, red arrows indicate soil C outputs, black arrows indicate direct pathways, grey arrows indicate indirect pathways.

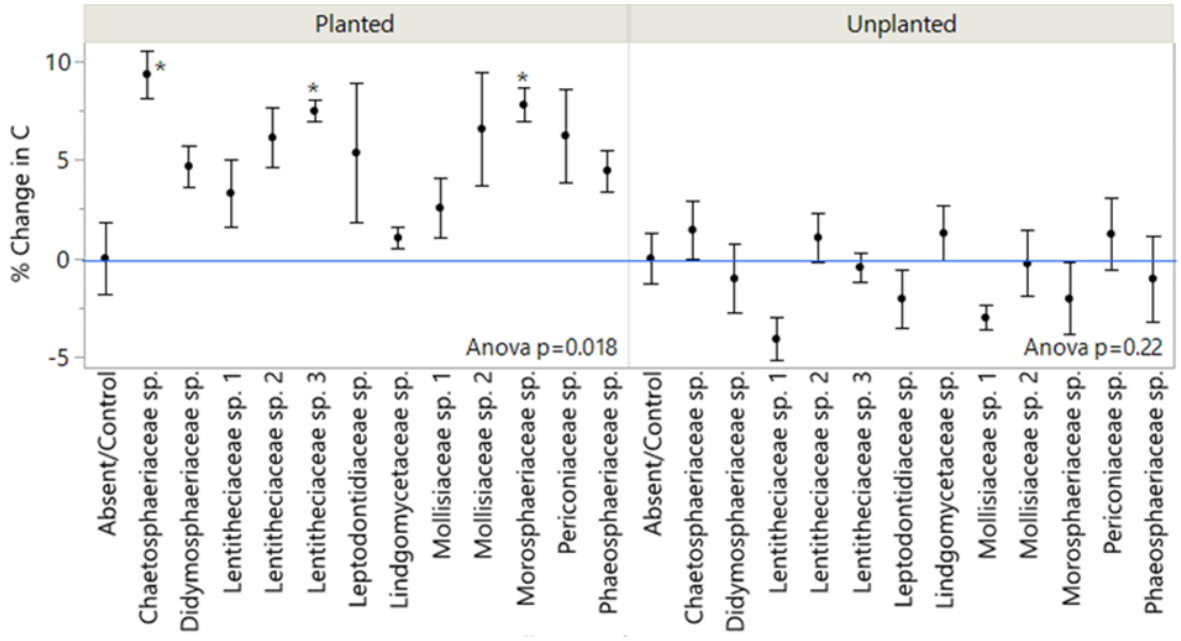

Figure 2. Changes in total soil C under inoculation with different fungal isolates compared to an uninoculated control. Values indicate percentage of change relative to mean of uninoculated control (blue line). Error bars indicate standard error, n=7 for inoculated treatments, n=6 for control. ANOVA results for planted and unplanted are presented. Asterisks indicate significant differences with control (Dunnett test, $p < 0.05$). C concentrations are presented in Table S2.

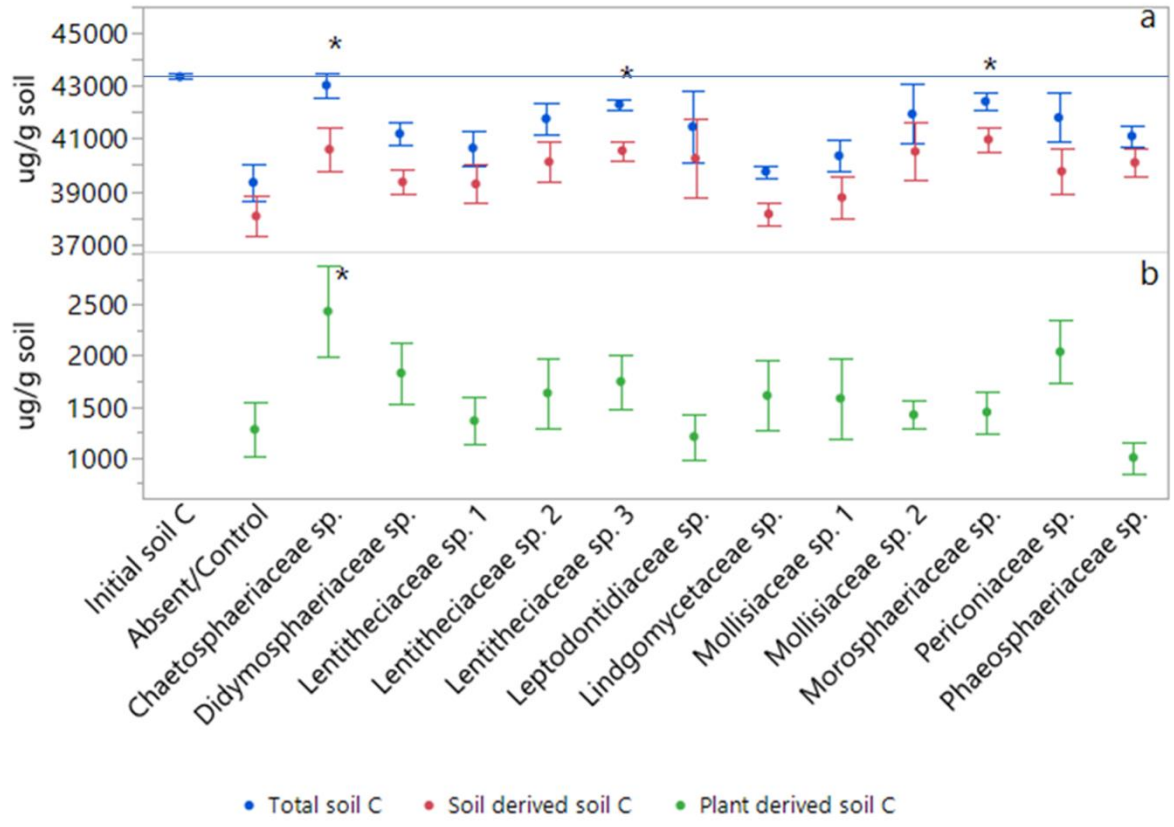

Figure 3. Concentrations of total soil C, soil-derived C (a) and plant-derived C (b), under inoculation with different fungal isolates or under no inoculation, and initial soil C pre-planting. Plant- and soil-derived C from C isotope partitioning. Error bars indicate standard error, n=7 for inoculated treatments, n=6 for uninoculated control. Note difference in axes scales. Asterisks indicate significant differences with control (Dunnett test, $p < 0.1$).

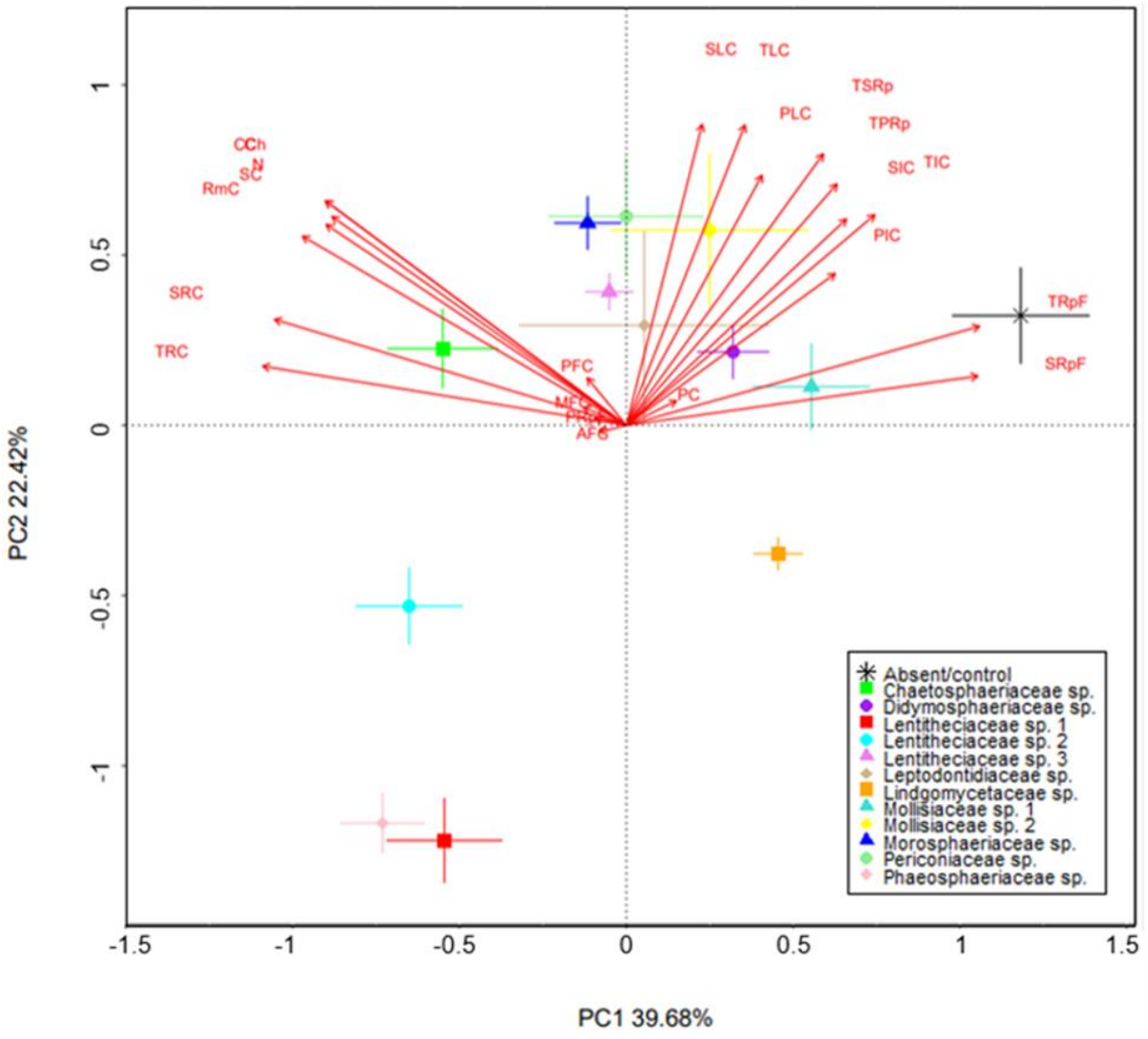

Figure 4. Fungi-dependent increases in soil C largely relate to measures for soil C stability. Principal component analysis (PCA) showing the soil C properties (red text) associated with the various fungal isolates (symbols). Soil C properties were measured via isotope analysis, soil incubations, and fractionation analysis of soil from wheat experiment. Soil C property abbreviations: AFC, aggregate C fraction %C; C, %C; CCh, % change in C (relative to average of control); MFC, MAOM fraction %C; N, %N; PC, plant-derived C (µg/g soil); PFC, POM fraction – %C; PIC, plant-derived intermediate C (µg C/g soil); PLC, plant-derived labile C (µg C/g soil); PRpF, plant-derived C respired fraction; RmC, estimated remaining C (µg/g soil); SC, soil-derived C (µg/g soil); SIC, soil-derived intermediate C (µg

C/g soil); SLC, soil-derived labile C (µg C/g soil); SRC, soil-derived resistant C (µg C/g soil); SRpF, soil-derived C respired fraction; TIC, total intermediate C (µg/g soil); TLC, total labile C (µg/g soil); TPRp, total plant-derived respired (µg/g soil); TRC, total resistant C (µg/g soil); TRpF, total C respired fraction; TSRp, total soil-derived C respired (µg/g soil).

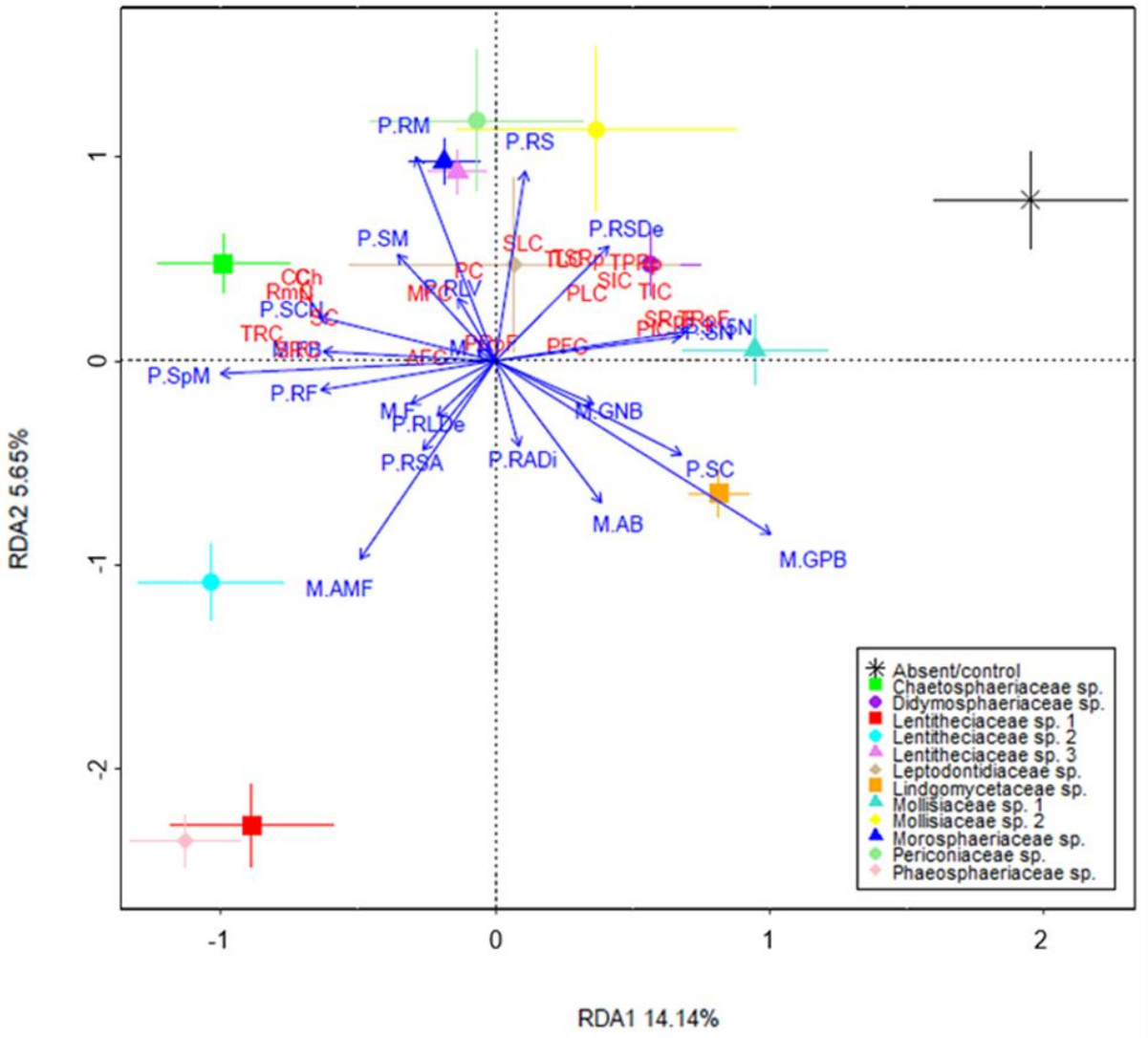

Figure 5. Fungal treatments resulting in increased soil C and its stability are associated with plant growth. Redundancy analysis (RDA) showing the microbial community and plant variables (blue text) driving changes in soil C properties (red text) associated with the various fungal isolates (symbols). Soil C properties were measured via isotope analysis, soil incubations, and fractionation analysis of soil from wheat experiment. Microbial community

and plant variables were measured using samples harvested from the wheat experiment. Microbial community (M.) and plant (P.) variable abbreviations: M.AB, actinobacteria (% of total community); M.AMF, arbuscular mycorrhizal fungi (% of total community); M.F, fungi (% of total community); M.FB, fungal to bacterial biomass ratio; M.GNB, gram negative bacteria (% of total community); M.GPB, gram positive bacteria (% of total community); M.TC, total community size (µg PLFA/g soil); P.RADi, root average diameter (mm); P.RF, root fork number (/g); P.RLDe, root length density (cm/g); P.RLV, root length per volume (cm/m^3); P.RM, root mass (g); P.RS, root/shoot ratio; P.RSA, root specific surface area (cm^2/g); P.RSDe, root specific density (g/cm^3); P.S15N, shoot δ15N (‰); P.SC, shoot %C; P.SCN, shoot C/N ratio; P.SM, shoot mass (g); P.SN, shoot %N; P.SpM, total spike mass (g). Soil C properties: AFC, aggregate C fraction – %C; C, %C; CCh, % change in C (relative to average of control); MFC, MAOM fraction – %C; N, %N; PC, plant-derived C (µg/g soil); PFC, POM fraction – %C; PIC, plant-derived intermediate C (µg C/g soil); PLC, plant-derived labile C (µg C/g soil); PRpF, plant-derived C respired fraction; RmC, estimated remaining C (µg/g soil); SC, soil-derived C (µg/g soil); SIC, soil-derived intermediate C (µg C/g soil); SLC, soil-derived labile C (µg C/g soil); SRC, soil-derived resistant C (µg C/g soil); SRpF, soil-derived C respired fraction; TIC, total intermediate C (µg/g soil); TLC, total labile C (µg/g soil); TPRp, total plant-derived respired (µg/g soil); TRC, total resistant C (µg/g soil); TRpF, total C respired fraction; TSRp, total soil-derived C respired (µg/g soil).

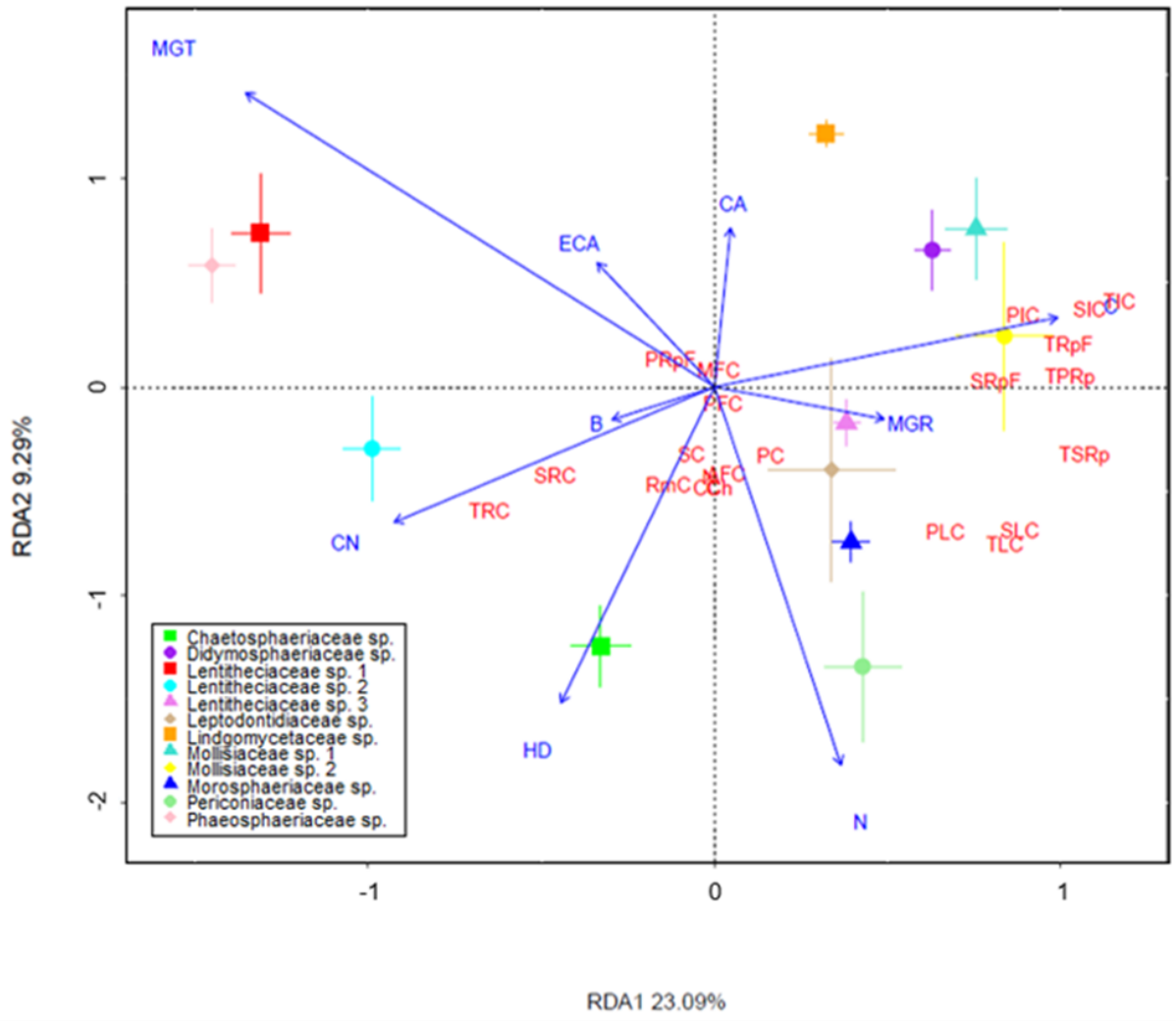

Figure 6. Fungal isolates involved in increased soil C and its stability have denser hyphae. Redundancy analysis (RDA) showing the fungal variables (blue text) driving changes in soil C properties (red text) associated with the various fungal isolates (symbols). Soil C properties were measured via isotope analysis, soil incubations, and fractionation analysis of soil from wheat experiment. Fungal variables were measured in an *in vitro* plate assay and values were averaged for the RDA. Fungal (F.) variable abbreviations: F.B, biomass (g); F.C, %C; F.CA, final colony area (cm^2); F.CN, C/N ratio; F.ECA, estimated final colony area (cm^2); F.HD, hyphal density (mg/cm^2); F.MGR, maximum growth rate (cm^2/day); F.MGT, time to maximum growth (days); F.N, %N. Soil C properties: AFC, aggregate C fraction – %C; C, %C; CCh, % change in C (relative to average of control); MFC, MAOM fraction – %C; N, %N; PC, plant-derived C (µg/g soil); PFC, POM fraction – %C; PIC, plant-derived

intermediate C (µg C/g soil); PLC, plant-derived labile C (µg C/g soil); PRpF, plant-derived C respired fraction; RmC, estimated remaining C (µg/g soil); SC, soil-derived C (µg/g soil); SIC, soil-derived intermediate C (µg C/g soil); SLC, soil-derived labile C (µg C/g soil); SRC, soil-derived resistant C (µg C/g soil); SRpF, soil-derived C respired fraction; TIC, total intermediate C (µg/g soil); TLC, total labile C (µg/g soil); TPRp, total plant-derived respired (µg/g soil); TRC, total resistant C (µg/g soil); TRpF, total C respired fraction; TSRp, total soil-derived C respired (µg/g soil).