



Environmental stress response limits microbial necromass contributions to soil organic carbon



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ARTICLE INFO

Article history:

Received 25 November 2014

Received in revised form

5 March 2015

Accepted 6 March 2015

Available online 23 March 2015

Keywords:

Necromass

Decomposition

Stabilization

Fungi

Soil organic carbon

Grazing

Stress response

ABSTRACT

The majority of dead organic material enters the soil carbon pool following initial incorporation into microbial biomass. The decomposition of microbial necromass carbon (C) is, therefore, an important process governing the balance between terrestrial and atmospheric C pools. We tested how abiotic stress (drought), biotic interactions (invertebrate grazing) and physical disturbance influence the biochemistry (C:N ratio and calcium oxalate production) of living fungal cells, and the subsequent stabilization of fungal-derived C after senescence. We traced the fate of ¹³C-labeled necromass from 'stressed' and 'unstressed' fungi into living soil microbes, dissolved organic carbon (DOC), total soil carbon and respired CO₂. All stressors stimulated the production of calcium oxalate crystals and enhanced the C:N ratios of living fungal mycelia, leading to the formation of 'recalcitrant' necromass. Although we were unable to detect consistent effects of stress on the mineralization rates of fungal necromass, a greater proportion of the non-stressed (labile) fungal necromass C was stabilised in soil. Our finding is consistent with the emerging understanding that recalcitrant material is entirely decomposed within soil, but incorporated less efficiently into living microbial biomass and, ultimately, into stable SOC.

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1. Introduction

Soil organic carbon (SOC) is the largest active carbon (C) pool in the terrestrial environment. The decomposition and formation of SOC are essential processes in determining SOC stocks, and interest in these processes has increased substantially in recent years due to their importance in global C cycling and associated feedbacks to climate change (Bellamy et al., 2005). It has historically been assumed that most of the C in stable SOC is directly plant-derived, but it is now accepted that a large proportion of organic material enters the soil C pool indirectly, following incorporation into microbial biomass (Kögel-Knabner, 2002; Liang and Balser, 2011; Liang et al., 2011; Miltner et al., 2012; Schimel and Schaeffer, 2012; Cotrufo et al., 2013). Although the potential for microbial cells to contribute to SOC formation has been recognised for several decades (McGill et al., 1975), the extent of these contributions is only recently becoming apparent. Living microbial biomass only

represents up to 1–2% of SOC, but the turnover of this biomass is a rapid, iterative process and so microbial necromass in mineral soils can ultimately contribute up to 50–80% of the C in stable SOC fractions (Simpson et al., 2007; Liang and Balser, 2011). Despite being widely acknowledged as a dominant pathway in the formation of stable SOC (Grandy and Neff, 2008; Kögel-Knabner et al., 2008; Cotrufo et al., 2013), the mechanisms governing microbial necromass decomposition, and subsequent incorporation into stable SOC, have received relatively little attention.

As with the traditional thinking in plant litter decomposition, the majority of microbially-derived SOC is assumed to originate from chemically or structurally 'recalcitrant' (complex or difficult to decompose) microbial components that are selectively avoided during decomposition (e.g. fungi with chitinous cell walls: Nakas and Klein 1979, Moore et al., 2005; Six et al., 2006). Litter chemistry (e.g. lignin concentrations or C:N ratios), and structural properties (e.g. leaf toughness or thickness) consistently emerge as the primary controls on plant decomposition rates (Melillo et al., 2002; Santiago, 2007; Dray et al., 2014), and it is not surprising that equivalent processes are expected to govern the breakdown of microbial necromass. An emerging paradigm, however, asserts that recalcitrant macromolecules are fully degraded, but less efficiently

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than labile material, and a greater proportion of recalcitrant C is thus lost through respiration, without being incorporated into decomposer biomass and ultimately into SOC (Lutzow et al., 2006; Cotrufo et al., 2013). Exploring the relative importance of these opposing mechanisms (selective preservation vs. reduced assimilation efficiency) for necromass mineralization and soil C stabilization has been highlighted as a high priority for ecosystem ecologists (Cotrufo et al., 2013).

As with plant litter, the biochemical structure (recalcitrance) of microbial necromass is a product of both constitutive and induced characteristics; taxonomic groups differ in their inherent biochemical composition (Six et al., 2006; Throckmorton et al., 2012), but can be altered drastically by both biotic and abiotic processes (Dijksterhuis and de Vries, 2006; Schimel et al., 2007). Although 'inherent recalcitrance' of microbial necromass is a strong determinant of initial mass loss (Sollins et al., 1996; Koide and Malcolm, 2009), a recent study suggests that microbial taxa cultured under similar conditions do not vary in their contributions to SOC formation (Throckmorton et al., 2012). However, all microbes *in situ* are subject to a variety of stressors (environmental stress, biotic antagonism and/or mechanical disturbance), which can alter the biochemical composition of cells, yet the effects of 'induced recalcitrance' on necromass stabilization remain unexplored.

A growing body of evidence highlights the importance of long-lasting effects in soil, where factors influencing the activity of living organisms can affect ecosystem functioning after cell death (e.g. Kostenko et al., 2012). Analogous to plant (Findlay et al., 1996) and animal (Hawlena et al., 2012) physiology, stress generally increases microbial C:N ratios as C demands rise to facilitate the synthesis of osmolytes, heat-shock proteins and structural defences (Schimel et al., 2007; Crowther et al., 2014). Many fungi, for example, increase the uptake of C, relative to N, to facilitate the synthesis of polyols (C-rich osmolytes), which allow fungal cells to maintain osmotic pressure during drought stress (Dijksterhuis and de Vries, 2006). Fungal investment in structural compounds has also been widely documented during biotic interactions and abiotic stress. For example, the stress-induced increases in the formation of calcium oxalate crystals, by-products of lignin decomposition, on the surface of fungal hyphae can serve as a physical barrier between living cells and the harsh local environment (Dutton et al., 1993). Such stress-induced changes in physiology and biochemistry have been proposed to limit the decomposition rates of plant and animal biomass (Findlay et al., 1996; Hawlena et al., 2012). Given the dominant role of microbial necromass decomposition in the formation of stabilized SOM, it is possible that similar changes might represent an important control on the balance between terrestrial and atmospheric carbon pools under current and future climate scenarios.

We explored the potential effects of stress on the C:N ratio and calcium oxalate crystal formation in saprotrophic fungi, and the consequent effects on microbial necromass decomposition and initial stabilization in soil. We grew two widespread fungal species, labeled with ^{13}C , in soil microcosms, and exposed them to a dominant abiotic stress (drought), biotic stress (isopod grazing) and mechanical disturbance (simulated by cutting). Following fungal death, we used a second set of soil microcosms to trace the fate of labeled C into living soil microbial biomass, dissolved organic carbon (DOC), mineralized (respired) C and total SOC. We tested the initial hypothesis that interactive biotic and abiotic stressors influence the C:N ratio and calcium oxalate production by fungal hyphae. We then tested the competing hypotheses that: (i) 'stressed' fungi contribute more C to SOC because of the selective preservation of recalcitrant macromolecules (Moore et al., 2005; Six et al., 2006); or (ii) 'unstressed' fungi will contribute more C

to SOC because of the reduced efficiency of microbes degrading 'stresses' (recalcitrant) necromass (Lutzow et al., 2006; Cotrufo et al., 2013).

2. Materials and methods

2.1. Overview of study design

Two cord-forming basidiomycete fungi, *Phanerochaete velutina* (DC.: Pers.) and *Resinicium bicolor* (Abertini and Schwein.: Fr.) (Cardiff University Fungal Genetic Source Collection), were selected due to their global distribution and contrasting responses to biotic and abiotic stress: *R. bicolor* is highly combative and shows reduced growth and enzyme production following temperature or grazing stress, whilst *P. velutina* is less combative but displays increased growth and enzyme production following stress (Crowther et al., 2012). These fungi were grown on ^{13}C -labeled soil with water potentials of either -0.006 or -0.06 MPa to replicate optimal and drought conditions, respectively. Isopod grazing (*grazing*), a dominant biotic control on fungal communities in temperate woodland ecosystems (Crowther et al., 2013), was also used as a stress, as was physical cutting (*cutting*), to simulate physical soil disturbance. These stressors and un-disturbed control treatments were each replicated five times per taxon across both moisture conditions (2 fungi \times 2 moisture conditions \times 3 disturbance treatments \times 5 replicates = 60 microcosms). Mycelia from stressed and unstressed environments were then harvested from the soil surface, added to soil within a second set of microcosms (60 centrifuge tubes containing fresh soil) so that fungal-derived C could be traced into (i) living microbial C, (ii) dissolved organic C, (iii) total soil C and (iv) respired C.

2.2. Fungal culturing and microcosm preparation

Both fungi were subcultured onto beech wood blocks (2 \times 2 \times 1 cm) within non-vented 9-cm dia. Petri dishes on 2% malt extract agar (MEA; 15 g L $^{-1}$ Lab M agar no. 2, 20 g L $^{-1}$ Munton and Fiston malt). Petri dishes were incubated in the dark at a constant temperature of 20 °C for 3 months prior to experimental use.

Soil microcosms were prepared following Crowther et al. (2011b). Briefly, loamy soil (pH: 5.52, % C: 11.57, % N: 0.63, % sand: 89.2, % silt: 4.1, % clay: 6.7%) was collected from temperate deciduous woodland (Yale-Myers Forest; 41° 57' 7.8" N, -72° 7' 29.1 W") to a depth of 10 cm and sieved on site through a 10 mm mesh. Sieved soil was air-dried in plastic trays and sieved again through 2-mm mesh before being frozen overnight at -20 °C to kill any remaining fauna. Prior to use, soil was re-wetted with 400 or 200 mL DH $_2$ O kg soil $^{-1}$, giving final water potentials of -0.006 and -0.06 MPa for optimal and drought treatments, respectively. Moistened soil (200 g) was then compacted to a depth of 5 mm within 34 \times 34 cm bioassay dishes and smoothed to provide a flat surface for fungi to grow into. Fungal-colonised wood blocks were then inoculated centrally onto the surface of the soil microcosms so that mycelial cords would emerge and grow across the soil surface.

All fungi were labeled by adding 0.269 mL of a 0.1 M solution (to avoid toxic effects of high glucose concentrations) of ^{13}C -labeled (99 atom %) glucose to soil, 5 mm ahead of the growing mycelial front. The solution was added immediately following mycelial emergence from wood blocks and repeated daily for a week to promote gradual incorporation throughout the mycelial system. Mycelia were then allowed to grow for 2 weeks to allow uniform labeling throughout each fungal system (Tordoff et al., 2011), before mycelia reached the edges of the dishes (which might have induced unintentional stress).

2.3. Stress treatments

Drought was induced via the differential re-wetting of soil (see above). Grazing and cutting treatments were then replicated across optimal and drought treatments.

The isopod *Porcellio scaber* (obtained from Carolina Biological Supplies) was used for the grazing treatment. Individuals were maintained in 2-L plastic pots containing compost. All containers were stored in the dark at 20 °C and moistened weekly using deionised water (DH₂O). Five individuals were introduced to each microcosm providing a grazer density of 83 m⁻² (falling within the range of field densities reported for temperate woodlands (Crowther et al., 2011b)). Isopods were removed after 2 days of grazing to prevent the decimation of entire fungal systems.

Mechanical disturbance was replicated by cutting. A sterilized scalpel was used to remove mycelia from the soil surface. This was done in a uniform, circular pattern around the central wood block, with an intensity to replicate the damage caused by isopod grazing. This was done to maintain equivalent stress levels across treatments allowing us to distinguish between the direct (physical damage) and indirect (saliva and grazing style) effects of grazing (i.e. the difference between 'grazing' and 'cutting' treatments).

2.4. Mycelial harvesting and chemical analyses

Mycelial cords were removed from the surface of the soil trays using a sterilized scalpel, cleaned with DH₂O, dried to constant weight and homogenized (ground to a fine powder) using a mortar and pestle. Each replicate was analyzed for total C, N and the ¹³C contents using a Costech ESC 4010 Elemental Analyzer (Costech Analytical Technologies Inc., Valencia, CA) coupled to a Thermo Delta^{Plus} Advantage (San Jose, CA, USA) continuous-flow isotope ratio mass spectrometer. A second sample was used for high-pressure liquid chromatography (HPLC) to determine calcium oxalate concentrations (see Supplementary Information for details). Remaining biomass (15 mg for *P. velutina* and 6 mg for *R. bicolor*) was then added to 50 mL centrifuge tubes for the decomposition assays.

2.5. Decomposition assays

The second set of microcosms contained 8 g of soil, that had been sieved (2-mm sieve), homogenized and moistened to within 70% water holding capacity (the optimum range for soil microbial activity). Biomass from each of the first set of microcosms was transferred to an individual second microcosm, retaining the n of 60. Decomposition assays were then conducted at 20 °C for 12 weeks, based on the time required for fungal necromass components to become stabilized in soil in previous studies (Sollins et al., 1996). Although more time is generally required for the long-term stabilization of plant-derived C, the relatively rapid turnover rates of fungal necromass (Sollins et al., 1996; Koide and Malcolm, 2009) meant that this time period could provide an estimate of initial stabilization rates, whilst avoiding the negative effects of long-term soil incubation (associated with constant changes in water content and loss of C and N). Our estimate of stabilization then should be considered in terms of 'initial' stabilization dynamics. This second-set of microcosms remained uncapped throughout the experiment to prevent CO₂ build up during incubation.

The mineralization rate of ¹³C-label was estimated, in real time, using a flow-through chamber technique. Gas samples from each replicate were monitored for 15 min each using cavity ring-down spectroscopy (CRDS; Picarro Inc., Santa Clara, CA, USA; Model: G1101-i). CRDS is a highly sensitive optical spectroscopic technique that enables measurement of absolute optical extinction by

samples that scatter and absorb light. The CRDS enabled us to simultaneously track total soil respiration and the δ¹³C of this respiration. Preliminary tests indicated that there was a peak in respired ¹³C, reaching maximum levels at approximately 48 h following necromass addition, that generally approached pre-addition levels within 6 days. We therefore estimated the rate of ¹³C mineralization at days 0, 2, 14, 42 and 84 following necromass addition, and calculated the area under the curve to represent total ¹³C respiration. The contribution of ¹³C-labeled necromass to total soil respiration was then estimated using the isotope mixing equation below. Although we did not expect high levels of fractionation in the microbial respiration of such highly-labeled necromass, we accounted for this potential in the mixing equation, using ¹³C respiration measurements from soil-only and fungus-only controls for each time point.

proportion necromass – derived

$$C = (\text{at\% } ^{13}\text{C mixture CO}_2 - \text{at\% } ^{13}\text{C soil}) / (\text{at\% } ^{13}\text{C necromass} - \text{at\% } ^{13}\text{C soil}) \quad (1)$$

Following the 12-week decomposition incubations, the soil from each microcosm was mixed separately and sampled for soil analyses. An aliquot (6 g) of each sample was used for chloroform-fumigation extraction to determine total microbial biomass C and dissolved organic C (see Supplementary Material). Another sample of dry soil (15 mg) was also extracted and ball-milled so that total C and the ¹³C contents could be determined using the Elemental Analyzer coupled to the GC-IRMS (see above). As homogenization disrupted the soil structure and aggregate formation, we did not explore differences in stabilization between soil C fractions (e.g. heavy vs. light), instead focusing on total soil ¹³C (Throckmorton et al., 2012). This gross estimate of total ¹³C provides an initial estimate of the C remaining in soil immediately following the first step of necromass decomposition (i.e. before repeated incorporation and turnover within multiple generations of living soil microbes). The concentration of ¹³C-label remaining in bulk soil, living microbial biomass, dissolved organic C and mineralized air was then calculated using Eq. (1), and expressed as a proportion of the initial ¹³C-labeled necromass added to each microcosm.

2.6. Statistical analyses

All statistical analyses were conducted in R version 3.0.3 (R Core Team, 2013). General Linear Models (GLMs) were constructed for all fungal biochemical characteristics (%C, C:N ratio and calcium oxalate content) and ¹³C data (concentrations in living microbial biomass, DOC, respired air and total soil C), with each 'fungus', 'drought', 'grazing' and 'cutting' included as factors. Second order interaction terms were also included to explore whether the effects of grazing and cutting varied across drought treatments, and across fungal species. Planned comparisons (contrast function) were then used to test for significant differences between specific treatments. GLMs were also used to investigate which biochemical properties best explained the percentage of necromass C stabilization in soil. Global models were fitted, including all biochemical data as factors (%C, %N, C:N and CaOx). Model selection was performed using the *dredge* function within R's MuMIn package (version 1.9.13; Bartoń, 2013) to identify the most plausible subset of models, ranked by Akaike Information Criterion (AICc) values. Where collinearity was detected between variables, each variable was modeled individually, and the strongest predictor was used as an indicator variable. Residuals from all models were checked for normality and homogeneity of variance following Crawley (2007).

3. Results

3.1. Fungal chemical composition

The stressors differentially influenced fungal CaOx concentrations, % C, and the C:N ratio of fungal necromass (Table 1). Grazing and physical disturbance significantly ($P < 0.05$) increased CaOx concentrations (Fig. 1). Drought also increased CaOx concentrations, although the magnitude of this effect varied depending on the fungal species (with greater effects in *R. bicolor* than *P. velutina*) and the presence of invertebrate grazers (Table 1). As with CaOx, all three stressors influenced the C concentrations in fungal cells, although the effects of drought, grazing, and cutting all varied significantly ($P < 0.05$) across fungi, with the magnitude of effects being greater in *R. bicolor* than *P. velutina* (Table 1). Some of these changes were reflected in differences in C:N ratio; both grazing and drought significantly enhanced fungal C:N ratios, although the effect of drought was significantly ($P < 0.05$) greater in *R. bicolor* than *P. velutina*. Unlike with % C, there was no overall effect of cutting on the C:N ratios of fungal necromass (Table 1) (see Fig. 2).

3.2. Decomposition assays and tracing ^{13}C label within microcosms

Following the decomposition assay, we were unable to account for all of the ^{13}C label added to most microcosms (Fig. 3). It is likely that this excess ^{13}C was mineralized between day 2 and 14, and hence not detected with our temporal sampling regime. Nevertheless, the proportion of ^{13}C label remaining in bulk soil represents a robust estimate of the initial stabilization of fungal-derived C. In the final model the proportion of ^{13}C label was significantly reduced by drought ($F_{1,56} = 15.35$; $P < 0.001$) and invertebrate grazing ($F_{1,56} = 5.83$; $P = 0.019$), whilst the effect of physical cutting only trended towards significance ($F_{1,56} = 3.37$; $P = 0.071$). These effects were all consistent across fungi and moisture regimes (Table 2). Approximately 40% of non-stressed *R. bicolor* biomass C remained in the soil following decomposition, but this fell to a mean of 22% when individuals were grown under stressful conditions. Similarly, approximately 31% of non-stressed *P. velutina* was stabilized in soil, but the mean value also fell to approximately 22% following environmental stress (Fig. 3; 4).

The patterns of ^{13}C label remaining in living microbial biomass and DOC were less consistent than in the bulk soil (Table 2). The proportion of microbial necromass ^{13}C detected in living microbial biomass differed significantly ($F_{1,56} = 14.15$; $P < 0.001$) between fungi, with greater contributions from *R. bicolor* than *P. velutina*, although this difference was mitigated by the presence of grazers (grazing*fungus: $F_{1,56} = 5.16$; $P = 0.027$). There were no significant ($P < 0.05$) effects of any treatments on the proportion of ^{13}C label remaining in DOC (Fig. 3).

The proportion of labeled C in respired air was significantly reduced by grazing ($F_{1,55} = 5.43$; $P = 0.024$), although this effect differed significantly ($F_{1,55} = 7.56$; $P = 0.01$) between fungi, with greater effects in microcosms containing *R. bicolor* than *P. velutina* (Fig. 4). However, as we were unable to account for all of the respired ^{13}C , these effects may simply be indicative of differences in initial (2 days) respiration rates, and cannot account for overall differences in ^{13}C mineralization. Indeed, given that grazing increased the total loss of ^{13}C from the soil, it is likely that the proportion of mineralized ^{13}C increased beyond that of un-grazed controls over the full course of the experiment.

3.3. Biochemical controls on decomposition

To explore the potential biochemical controls on necromass stabilization, we regressed '% ^{13}C stabilized in soil' against biochemical data (%C, %N, C:N and calcium oxalate). For *P. velutina* only calcium oxalate correlated significantly ($F_{1,26} = 10.82$; $P = 0.002$), and negatively with initial C stabilization rates. For *R. bicolor*, calcium oxalate content was co-linear with C:N ratio ($R^2 = 0.12$) and %C ($R^2 = 0.12$). However, within individual models, calcium oxalate content had by far the strongest relationship with necromass stabilization ($F_{1,26} = 12.48$, $P = 0.002$), so this variable was selected as the indicator variable for chemical recalcitrance, explaining approximately 35% of the variation in initial SOC formation (Fig. 5).

4. Discussion

Identifying the dominant processes governing SOC formation is essential to our understanding of soil nutrient dynamics, fertility and C cycle feedbacks to climate change (Bellamy et al., 2005; Liang and Balser, 2011). Although various climatic and edaphic characteristics are known to influence the formation of SOM (Cotrufo et al., 2013), we used controlled laboratory conditions to isolate the effects microbial biochemistry on initial C stabilization. By altering the biochemistry of fungal cells, environmental stress and biotic interactions can influence long-term C dynamics in soil. This finding is in stark contrast to those of a recent field study, where considerable differences in inherent microbial recalcitrance had a negligible effect on necromass contributions to stabilized soil C (Throckmorton et al., 2012). It is likely that the effects of microbial biochemical composition were obscured by the noise associated with environmental variability under complex field scenarios. By controlling for this environmental variation our microcosm study highlights that stress-induced changes in biochemistry can reduce fungal C contributions by up to 18%. As with plants (Findlay et al., 1996) and animals (Hawlena et al., 2012), stress can alter the biochemical composition of microbial necromass, in this case driving increases in fungal C:N ratios and calcium oxalate crystal

Table 1
Statistical outputs from final models testing the effects of environmental stressors on the calcium oxalate (CaOx) concentrations, %C and C:N ratio. Best models were selected using the *dredge* function in R. Terms with missing values were dropped from the final model based on AIC values.

	CaOx		%C		CN	
	F _(DF)	P	F _(DF)	P	F _(DF)	P
Drought	19.62 _(1,52)	<0.001	1.75 _(1,52)	0.19	62.70 _(1,52)	<0.001
Grazing	12.26 _(1,52)	0.001	3.96 _(1,52)	0.05	15.81 _(1,52)	<0.001
Cutting	12.07 _(1,52)	0.001	13.46 _(1,52)	<0.001	0.35 _(1,52)	0.56
Drought*grazing	5.08 _(1,52)	0.03	–	–	–	–
Drought*cutting	–	–	–	–	–	–
Drought*fungus	5.87 _(1,52)	0.02	24.22 _(1,52)	<0.001	4.42 _(1,52)	0.04
Grazing:fungus	–	–	19.81 _(1,52)	<0.001	–	–
Cutting:fungus	–	–	4.82 _(1,52)	0.03	–	–

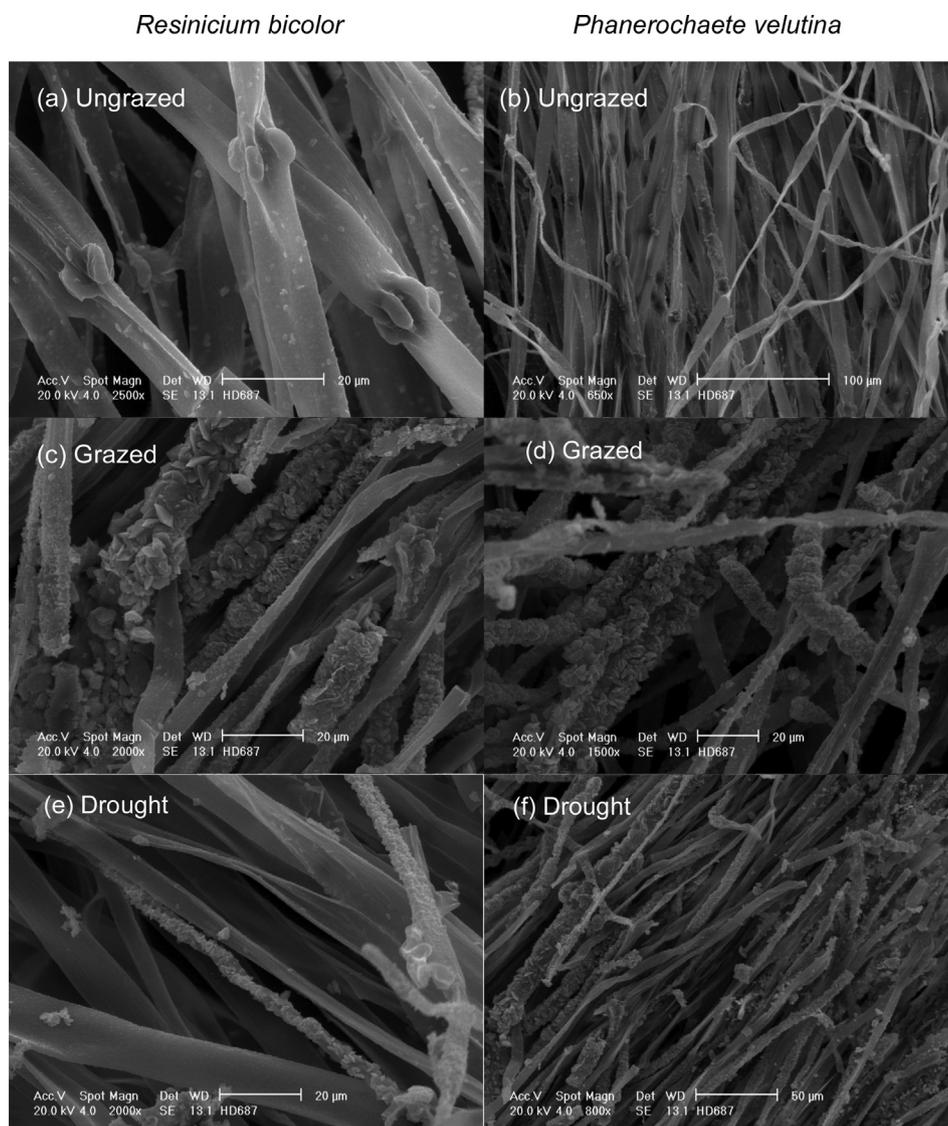


Fig. 1. Electron scanning microscope images of mycelial cords of calcium oxalate crystal production on the cords of *Resinicium bicolor* (a, c, e) and *Phanerochaete velutina* (b, d, f) following growth under optimal conditions (a, b), isopod grazing (c, d) and drought (e, f). Images show that the density and size of crystals accumulated increase during stress.

production. These biochemical changes are likely to have increased the physical and chemical recalcitrance of fungal cells. However, in contrast to traditional expectations that the more recalcitrant material would be selectively avoided during the decomposition process (Moore et al., 2005; Six et al., 2006), the induced recalcitrance ultimately increased losses of fungal-derived C from soil. Our finding is then consistent with the understanding that more recalcitrant material is incorporated less efficiently into microbial biomass and. Ultimately, less C is retained within the soil (Lutzow et al., 2006; Cotrufo et al., 2013). We emphasize that the time-scale of our study only allows us to assess the initial stabilisation of fungal-derived C in SOC (Sollins et al., 1996). Whether these mechanisms translate to longer-term SOC stabilization dynamics should be tested under field conditions over long (year to multi-decadal) timescales.

The 'legacy' (long-lasting) effects of biotic interactions in soils have gained attention of late, due to the potentially large effects on ecosystem functioning (e.g. Kostenko et al., 2012). Soil fauna are known to influence the immediate mineralization and stabilization of soil C during ingestion, by making organic material available to gut microbes, and following excretion, by changing soil structure

and stimulating microbial activity within faecal deposits (Wolters, 2000). We highlight the potential for a new mechanism, whereby fauna can alter the biochemical structure of living microbial cells and, consequently, the stabilization of microbial products after cell death. Similarly, it is widely acknowledged that abiotic conditions (e.g. temperature and drought) directly influence the turnover of C and nutrients in soil by regulating the metabolic activity and community compositions of living microbes (Schimel et al., 2007; Crowther and Bradford, 2013). Our data highlight that, along with these simultaneous effects of environmental conditions, climate-induced changes in microbial physiology can also have subsequent consequences for the mineralization and stabilization of C long after cell death.

The effects of stress on initial C stabilization in soil did not vary significantly between the two fungal species. Although these fungi display opposing growth (Crowther et al., 2011a) and enzymatic (Crowther et al., 2012) responses to grazer stress, they displayed similar directional changes in calcium oxalate production and C:N ratio during stress, effects that ultimately reduced necromass contributions to SOC. It is possible that such similar outcomes arise in different fungi because of a shared 'environmental stress

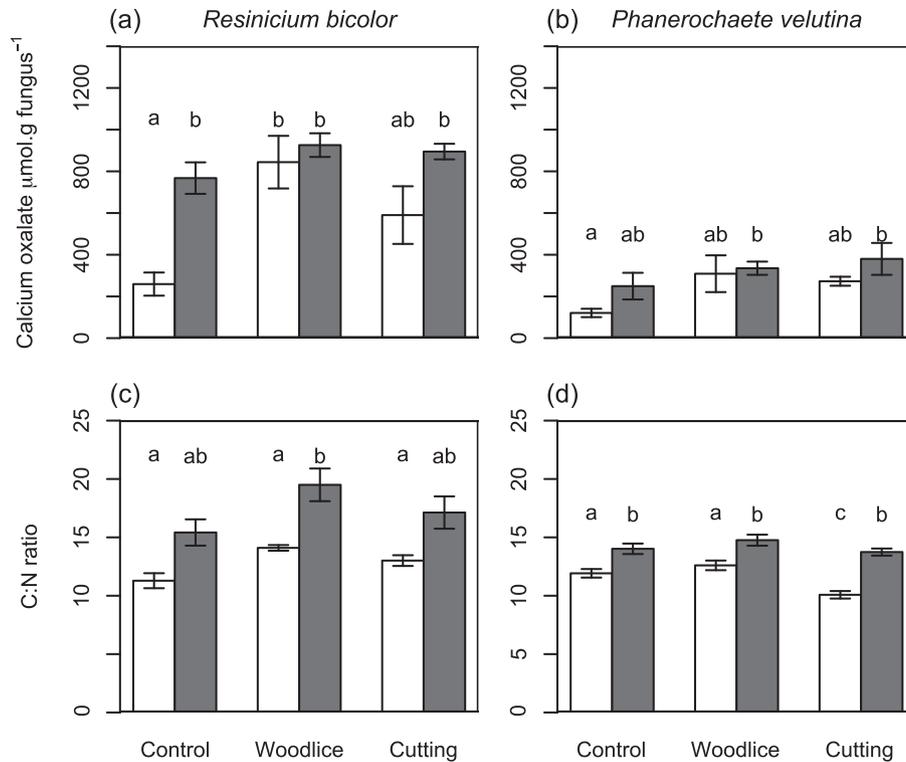


Fig. 2. Effects of abiotic, biotic and mechanical stress on the calcium oxalate production (a, b) and C:N ratio (c, d) by *Resinicium bicolor* (a, c) and *Phanerochaete velutina* (b, d) growing in compacted soil microcosms. White and gray bars represent fungi grown under optimal (-0.006 MPa) and drought (-0.06 MPa) conditions, respectively. Different letters above bars refer to significantly (2 way ANOVA: $P > 0.05$) differences between treatments.

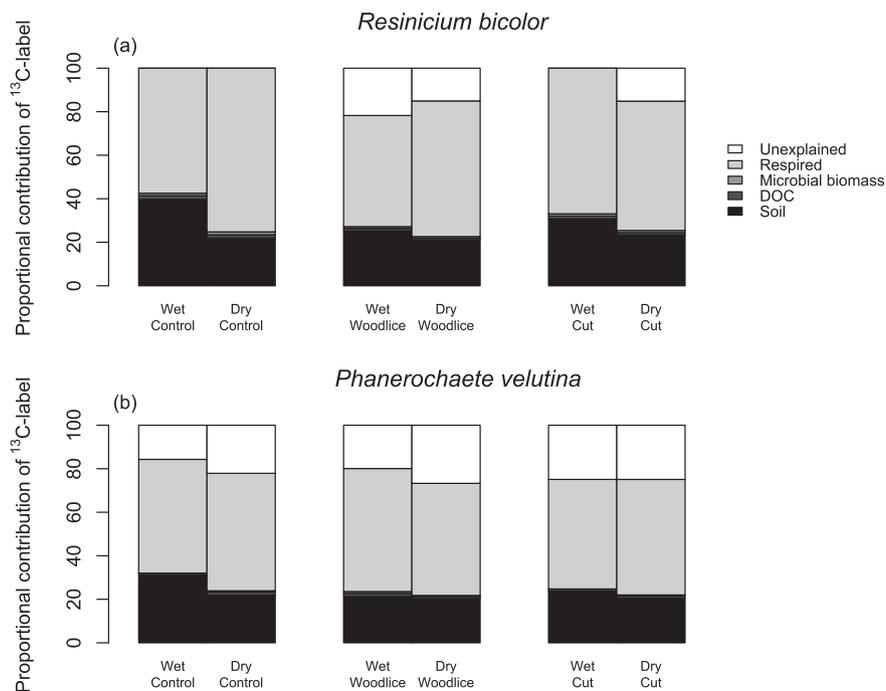


Fig. 3. Proportional contribution of ^{13}C -labeled *Resinicium bicolor* (a) and *Phanerochaete velutina* (b) to stabilized soil C, dissolved organic C, microbial biomass C and mineralized (respired) C within decomposition assays. White sections represent un-detected C. It is likely that this excess ^{13}C was mineralized between day 2 and 14, and not detected with our sampling regime.

response' (ESR) (Gasch, 2007). This common gene expression response has been conserved widely throughout the fungal kingdom and involves the activation of ~ 300 genes in response to a wide range of biotic and abiotic stresses (Gasch, 2007). It is possible

that such a common stress response might have similar implications for the stabilization of necromass C across a range of fungal species, especially those of widespread generalists like the current study species. Nevertheless, fungi display a huge diversity of

Table 2

Statistical outputs from final models testing the effects of environmental stressors on the proportion of ^{13}C -labeled fungal necromass remaining in bulk soil, mineralized air, dissolved organic C (DOC) and in living microbial biomass. Best models were selected using the *dredge* function in R. Terms with missing values were dropped from the final model based on AIC values.

	Soil C		Mineralized C		DOC		Microbial C	
	F _(DF)	P	F	P	F	P	F	P
Drought	15.35 _(1,56)	0.00	1.03 _(1,56)	0.31	–	–	–	–
Grazing	5.83 _(1,56)	0.02	5.43 _(1,56)	0.02	0.92 _(1,55)	0.34	0.08 _(1,55)	0.78
Cutting	3.37 _(1,56)	0.07	–	–	2.31 _(1,55)	0.13	–	–
Drought*grazing	–	–	–	–	–	–	–	–
Drought*cutting	–	–	–	–	–	–	–	–
Drought*fungus	–	–	–	–	–	–	–	–
Grazing:fungus	–	–	7.56 _(1,56)	0.01	–	–	5.16 _(1,55)	0.03
Cutting:fungus	–	–	–	–	–	–	–	–

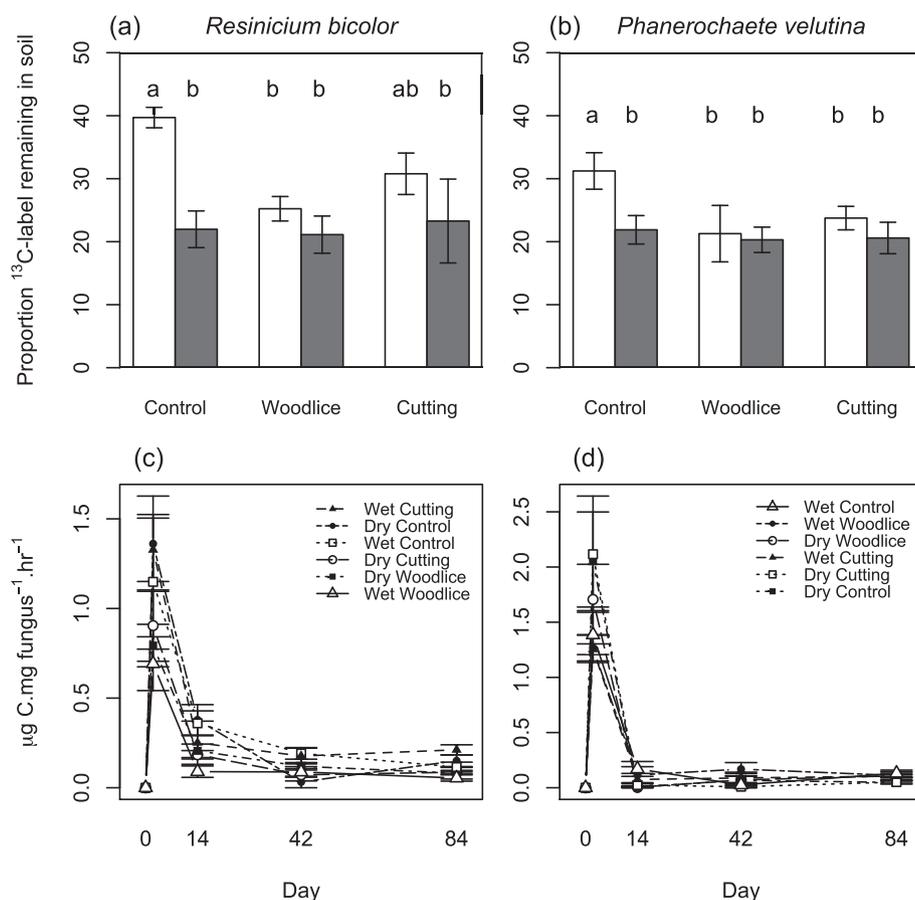


Fig. 4. Effects of abiotic, biotic and mechanical stress on the percentage of initial *Resinicium bicolor* (a) and *Phanerochaete velutina* (b) necromass stabilized within soil. White and gray bars represent the contributions of fungi grown under optimal (-0.006 MPa) and drought (-0.06 MPa) conditions, respectively. Also shows the mineralization rates of ^{13}C -labeled *Resinicium bicolor* (c) and *Phanerochaete velutina* (d) over 84 days following addition of necromass to soil microcosms on Day 1. Although initial mineralization rates of the most labile material tended to be higher than those of stressed necromass, more ^{13}C remained in the soil after 84 days.

biochemical and physiological responses to stress, and the efficiency of these responses can vary drastically across species (Crowther et al., 2014). The present study highlights the potential for stress responses to influence microbial C contributions to SOC. Exploring a wider range of fungal biochemical responses across various microbial taxa is now essential if we are to establish a thorough understanding of the mechanisms governing the stabilization of microbial necromass C in soil.

Of the measured fungal traits, calcium oxalate concentration was the strongest predictor of initial C stabilization rates. Higher levels of calcium oxalate production were consistently associated

with decreased fungal C contributions to total SOC. Production of oxalic acid, a by-product of lignin decomposition, has been shown to increase substantially in a wide range of basidiomycete fungi during unfavorable conditions (Shimada et al., 1997). This is precipitated as crystals of an insoluble salt, calcium oxalate, that line the outside of mycelial cords (Fig. 1). Accumulating crystals can form a physical barrier between cell surfaces and the outside environment, which has the potential to reduce water loss from fungal cells and minimise the effects of antagonistic soil organisms (Dutton et al., 1993). As predicted for plant litter (Findlay et al., 1996), it is likely that such physical protection restricted the

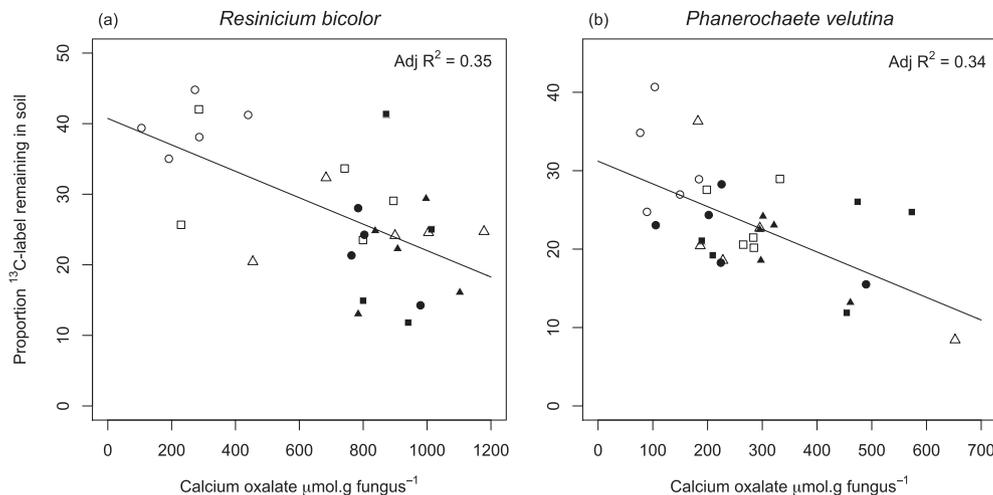


Fig. 5. Linear relationships between fungal calcium oxalate production and proportional necromass contributions to stabilized soil C. The negative effect of calcium oxalate production on initial SOC formation was equivalent between *Resinicium bicolor* (a) and *Phanerochaete velutina* (b). White and black symbols represent the contributions of fungi grown under optimal (-0.006 MPa) and drought (-0.06 MPa) conditions, respectively. Symbol shapes indicate different treatments (control: circles, grazing: triangles, and cutting: squares).

accessibility of fungal C to living microbes. If crystal formation reduced the efficiency of fungal-derived C assimilation by soil microbes, then a greater proportion is likely to have been lost to the atmosphere through respiration. Although the importance of calcium oxalate in regulating soil nutrient turnover and biogeochemical cycles has long been recognized (Graustein et al., 1977), the present study provides another mechanism by which calcium oxalate production might influence the balance between terrestrial and atmospheric C pools. However, along with calcium oxalate production, various other physiological stress responses are also likely to simultaneously influence the efficiency of necromass decomposition. Indeed, calcium oxalate concentration only explained 35% of the variation in initial C stabilisation rates in our fungi and a broad suite of other biochemical and structural changes are likely to have contributed to the changes observed. Increased melanin production under stressed conditions has, for example, been proposed to influence the decomposition rates of microbial necromass components in soil (Koide et al., 2014). Identifying the key biochemical traits that govern the decomposition rates of microbial cells is likely to provide a more mechanistic understanding of the environmental (biotic and abiotic) controls on the microbial C stabilization in soil (Crowther et al., 2014).

That environmental stress can influence the stabilization of microbial necromass C highlights another potential link between climate and soil C dynamics. Drought consistently emerges as a primary control on the activity (growth, C-use efficiency and decomposition rates) of terrestrial microbes (Schimel et al., 2007; Crowther et al., 2014). Re-allocation of energy from enzyme synthesis towards osmolyte production under drought stress is a dominant mechanism governing differences in C mineralization rates across landscapes (Manzoni et al., 2014). If increasing osmotic stress leads to greater fungal investment in C-rich molecules such as polyols and calcium oxalate, it is likely that necromass constituents will be increasingly recalcitrant in drier environments (Fernandez and Koide, 2013; Koide et al., 2014). The stabilization of microbial necromass C in soil might, therefore, track changes in C mineralization rates, decreasing along gradients of soil moisture. The legacy effects of microbial stress are also likely to have consequences for soil C feedbacks to climate change: as environmental conditions shift to the extremes of species tolerances and the frequency of extreme events increases, microbial stress is likely to limit the stabilization of microbially-derived C in soil. Such climate-

induced increases in microbial stress might have partially contributed to the broad-scale decreases in total SOC observed in recent decades (Bellamy et al., 2005).

5. Conclusions

The present study provides a novel mechanism by which environmental stress (biotic interactions, abiotic stress and mechanical disturbance) during the lifetime of an individual microbe, can influence the initial stabilization of its necromass C in soil. Although these stressors have a variety of effects on the partitioning of microbial necromass C between living decomposer biomass, DOC and respired CO_2 , they consistently reduced fungal contributions to total soil C. It is likely that fungal C contributions to SOC in the present study under-represent those observed under field conditions, as the homogenization of soil might have limited the formation of aggregates that restrict decomposer activity. Furthermore, increases in fungal exudates under stressed conditions might also contribute to changes in soil C stabilization. However, our study excluded other microbial products, focusing on the decomposition of the structural components of microbial cells under controlled laboratory conditions. In doing so, we are able to identify a potential mechanism linking environmental conditions with fungal contributions to soil C. That both fungal species showed similar trends – with reduced stabilization of ^{13}C -labeled necromass as stress-induced calcium oxalate production increased – highlights the potential generality in this process, at least across similar taxa. We stress that these results simply highlight the potential for environmental stress responses to influence the stabilization of microbial necromass C in soil. It is now important that we explore the biochemical mechanisms governing this process under field scenarios, due to the potential to explain differences in soil C storage across environments and to improve predictions about the strength of feedbacks between climate change and soil C efflux.

Statement of authorship

The study was designed by TWC and MAB. Practical work and analyses were performed by TWC, NS, EEO and DSM. Statistical analyses were performed by SMT and the manuscript was written by TWC.

Acknowledgments

This work was funded through fellowships to TWC by the Yale Climate & Energy Institute, and the British Ecological Society; and to MAB by a US National Science Foundation grant (DEB-1021098). Thanks to Michael Strickland for critical discussion at the early stages of the study.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.soilbio.2015.03.002>.

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