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Microspectroscopic visualization of how biochar elevates the soil organic carbon ceiling

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The soil carbon saturation concept suggests an upper limit to store soil organic carbon (SOC), set by the mechanisms that protect soil organic matter from decomposition. Biochar has the capacity to protect new C including rhizodeposits and microbial necromass. However, the decadal scale mechanisms by which biochar influences the molecular diversity, spatial heterogeneity, and temporal changes of SOC persistence remain unresolved. Here we show that the soil C saturation ceiling of a Ferralsol under subtropical pasture could be elevated by 2 Mg (new) C ha⁻¹ by the application of *Eucalyptus saligna* biochar 8.2 years after the first application. Using one, two-, and three-dimensional analyses, significant increases were observed in the spatial distribution of root-derived ¹³C in microaggregates (53-250 μm, 11 %) and new C protected in mineral fractions (<53 μm, 5 %). Microbial C-use efficiency was concomitantly improved by lowering specific enzyme activities, contributing to the decreased mineralization of native SOC by 18 %. We provide evidence that the global SOC ceiling can be elevated using biochar in Ferralsols by 0.01-0.1 Pg new C yr⁻¹.

Main

Human activities risk releasing 260 Pg of carbon (C) as carbon dioxide (CO₂) globally that is irrecoverable on a timescale relevant to avoiding profound climate impacts^{1,2}. Agricultural soils contribute an average of 2 Mg C lost ha⁻¹ yr⁻¹ globally³⁻⁵. It has been estimated that 122 Mg soil organic C (SOC) ha⁻¹ to 1 m depth has been lost over 1 Mha of land converted to tropical grasslands⁶, with 40 % of this area occurring on Ferralsols⁷. To meet the Paris Agreement of limiting global warming to below 2°C, the Intergovernmental Panel on Climate Change has shown that CO₂ removal (CDR) techniques are urgently needed^{8,9}.

Soil C management⁴⁻⁶ and the application of biochar¹⁰ are appealing CDRs^{9,11} as they also improve soil health, sustain agricultural productivity^{12,13}, and increase resilience of ecosystem services^{14,15}. Protecting and rebuilding soil C could draw down 5.5 Pg CO₂ yr⁻¹, representing 25 % of the potential of natural climate solutions to deliver CDR through conservation, restoration, and improved land

management practices⁶. However, there are biophysical and socio-economic barriers to CDR with SOC management^{4,6,16,17}.

Biochar is recognized as a CDR because of its persistence^{9,11} in the environment. The pyrolysis of biomass can deliver bioenergy outcomes, as well as agronomic and non-CO₂ greenhouse gas benefits through use of biochar as a soil amendment¹⁸⁻²². Biochar systems generally show life cycle climate change impacts of emissions reduction in the range of 0.4 -1.2 Mg CO₂e Mg⁻¹ dry feedstock²³.

Organo-mineral interactions can increase SOC persistence, in some cases over a millennial timescale^{24,25}. There is a general understanding of the effect of microbial activity²⁶ and mineral protection²⁷ on SOC storage. However, there are knowledge gaps on the contribution of molecular diversity of organic compounds, fine-scale spatial heterogeneity, and temporal variability in soil conditions. Such composition-space-time interactions influence the accessibility of decomposer communities to the substrate^{28,29}. Here, we propose a mechanism by which biochar acts as a bio-catalyst to accelerate the formation of organo-mineral and organo-organic interfaces in microaggregates (53-250 μ m) and mineral protection of SOC (Fig. 1). Biochar can sorb root-derived C (rhizodeposits) and form biofilms on its surfaces. The very fine layer of soil minerals that subsequently builds on the surfaces of biochar as it ages in soil³⁰⁻³² protects rhizodeposits from microbial metabolism^{33,34}, and at the same time incorporates microbial necromass³⁵⁻³⁸. This coating can desorb from the surface during aggregate turnover or in response to a change in soil conditions such as pH, redox and moisture³⁹. The rhizodeposits and microbial necromass are then captured in microaggregates^{35,40,41} (e.g. <250 μ m). A new coating can then form in its place. These processes repeat, building rhizodeposits in soil over time (Fig. 1). We examine these processes in detail to quantify the potential of biochar to elevate the SOC storage ceiling. To do this, we applied *Eucalyptus saligna* biochar (550°C) to a historic field site established in 2006⁴¹ (Ferralsol under managed subtropical pasture). The mechanisms (Fig. 1) that we tested included the negative priming via higher microbial C-use efficiency and restricted access to substrates, and enhanced mineral protection via

catalytic biochar surfaces. We demonstrate the importance of fine-scale spatial heterogeneity and temporal variability of diverse C functional groups in association with mineral fractions for building and protecting rhizodeposits over a decade.

Elevating SOC storage capacity

We hypothesize that biochar enhances the protective mechanisms for soil organic matter (SOM), and that a greater C storage capacity can therefore be obtained through strategic applications of biochar. The field site was converted to managed pasture from subtropical forest 100 years ago. This led to a loss of 17 % of the original soil C stock compared to the adjacent native rainforest (data not shown). To quantify elevated C storage capacity, we measured soil C stocks in the managed pasture over 9.5 years (Table S1) from four treatments: (1) biochar applied to a part of the historical biochar plots 8.2 years after the trial was established (“recent + historical”); (2) biochar applied to a part of the control plots 8.2 years after the trial was established (“recent”); (3) biochar applied 8.2 years previously (“historical”); and (4) nil biochar plots (“control”).

All field plots were managed via annual fertilizer application at the start of winter, coinciding with over sowing by annual ryegrass (Methods). The total soil C stock in the unamended pasture soil (control) did not change over 9.5 years^{41,42,43} (Fig. 2a; $P > 0.05$) and remained at 35 Mg C ha⁻¹ in the 0-100 mm layer when sampled at 8.2 and 9.5 years after the field trial was set-up. The original application of *Eucalyptus saligna* biochar (550°C) in 2006 resulted in a rapid increase in soil C to 40 Mg C ha⁻¹ (10 Mg biochar ha⁻¹, 76 % C, 7.6 Mg biochar-C) and SOC continued to increase, plateauing at 50 Mg C ha⁻¹ at 8.2 years (“historical”). When recent biochar amendment was incorporated into the plots at the same dose after 8.2 years following the original application (“recent + historical”), the C stock immediately increased from 50 to 56 Mg C ha⁻¹ (Fig. 2a) because of the C added from biochar. The C stock then continued to increase further to 58 Mg C ha⁻¹ between 8.2 and 9.5 years. This additional 2 Mg C ha⁻¹ was accumulated because of the increased protection mechanisms for new C provided by the biochar, thus elevating the C storage ceiling (Fig. 2a).

The additional 2 Mg new C ha⁻¹ (Fig. 2a) can be explained by negative priming of SOC mineralization (Fig. 2b). Comparing the net cumulative SOC mineralization where there was a recent application of biochar to the control (defined as priming in this study), the recent biochar amendment to the historical plots (“recent + historical”) lowered SOC mineralization (*i.e.* negative priming) by 89 g CO₂-C m⁻² compared with the recent biochar amendment to the control (“recent”) which lowered SOC mineralization by 55 g CO₂-C m⁻² ($P < 0.05$, Fig. 2b). Recent biochar initiated a small (3 g CO₂-C m⁻²) positive priming effect, whilst recent + historical biochar immediately triggered negative priming (Fig. 2b). Neither recent + historical or recent had an impact on total respiration or root respiration compared to the control ($P > 0.05$, Figs. S1 & S2). As a portion of the total CO₂ flux, root respiration remained relatively consistent (30-32 %) and was unaffected by treatments ($P > 0.05$, Table S2).

The additional 2 Mg C ha⁻¹ that accumulated between 8.2 and 9.5 years because of the recent application of biochar to the historical biochar plots (“recent + historical”) accrued through the stabilization of rhizodeposits and microbial necromass (Fig. 3). Recent + historical biochar had a similar proportion of total recovered ¹³C (58 ± 5.7 %, Fig. 3a) compared to the historical biochar plots (“historical”) (60 ± 9.8 %), with this being around 18 % greater than the control (42 ± 7.3 %) and the recent biochar amendment (“recent”) (45 ± 4.5 %; Fig. 3b) after the pulse-labelling event at 9.5 years ($P < 0.05$; Table S3). The increase in belowground ¹³C recovery could be largely explained by an increase in mineral-protected soil organic matter (M-SOM) associated ¹³C (14 %, $P < 0.05$, Table S4). Initially, recent + historical biochar nearly doubled the ¹³C retention in the occluded particulate organic matter (O-POM) fractions (5 mg ¹³C m⁻²) of microaggregates (< 250 μm) and M-SOM fractions (14 mg ¹³C m⁻²) of macroaggregates (250-2000 μm) at 8.9 years compared to the recent biochar (Figs. S3a & b). The root-derived ¹³C from rhizodeposition was accumulated gradually into O-POM in macroaggregates and M-SOM at 9.2 years (Figs. S3c & d), which was in turn transformed into M-SOM fractions in micro- and macroaggregates by 9.5 years (Figs. S3 e & f).

Microbial contribution and responses to stabilization of rhizodeposits

To determine the microbial contribution to the increased SOC storage capacity, we quantified catabolic enzyme activities, metabolic quotient of native SOC (bulk soil) and rhizodeposition (^{13}C content), and specific enzyme activity (the ratio of enzyme activity-to-microbial biomass) comparing a recent biochar amendment to the control (“recent”) and to the historical biochar plots (“recent + historical”). Microbial biomass was increased by 11 % in the recent + historical biochar compared to the recent biochar between 8.9 and 9.5 years (Table S5a). This might result from the stimulation of microbial co-metabolism³¹ of biochar-C, root-C and SOC which induced initial positive priming in the recent biochar amendment (Fig. 2b). The recent + historical biochar had increased substrate-induced respiration for citric, oxalic and malic acids compared to the recent biochar (Microresp, Fig. S4; Table S6). No differences were detected for 12 other substrates. This greater respiration induced by carboxylic acids (*e.g.* root exudates) may partially explain the higher metabolic quotient associated with bulk SOC and rhizodeposition in the recent biochar *cf.* the recent + historical biochar (Table S5b & c). The recent + historical biochar might result in higher substrate-use efficiency which supports an earlier establishment of negative priming compared to the recent biochar (Fig. 2b). It was previously shown that the recent biochar significantly increased bacterial diversity and the relative abundance of nitrifiers and bacteria consuming biochar C after one year, but the soil bacterial communities from the recent + historical plots did not differ from the control³³. This suggests that the microbial accessibility to SOM might be limited in the recent + historical biochar plots, whereas in the recent biochar, the soil microorganisms had to cope with changes in C-substrate type and availability⁴⁵.

The ratio of enzyme activities to total microbial biomass was lower in both recent + historical biochar and recent biochar compared to the control (Table S7) despite no difference in enzyme activities (Table S8). This suggests that for a given amount of microbial biomass, less enzymes were produced in the biochar-amended soil. A low ratio of extracellular enzymes to microbial biomass can slow down the degradation of native SOC⁴⁶. This is consistent with increased microbial C-use efficiency (Tables S4b & c), which may indirectly contribute to the negative priming. It has been suggested that the presence of opportunistic microbes that meet their energy and nutrient demands by exploiting the

catalytic activities of decomposers could lower the specific enzyme activity⁴⁶. The spatial arrangement between microbes and substrates is critical to this process.

Spatial examination of organo-mineral interactions

To better understand the process of negative priming following biochar application, we examined whether the stabilization of rhizodeposits may be facilitated via protection with the abundant Fe and Al in soil. Root exudates can interact with Fe oxides in the soil to promote the formation organo-mineral complexes. Aluminum may also protect root-C from biodegradation. The development of organo-mineral complexes was assessed using three-dimensional focused ion beam (FIB) coupled with scanning electron microscopy (SEM) with energy dispersive X-ray spectroscopy (3D-FIB-SEM-EDS) on intact representative soil aggregates from the recent + historical biochar (Fig. 3c) where C retention co-located with clay minerals, but, not in the recent biochar (Fig. 3d).

To provide a deeper insight into the molecular diversity of organic compounds and the temporal and lateral arrangement with respect to organo-mineral interfaces, we conducted *in situ* spectromicroscopic analysis of free water-stable microaggregates (53-250 μm) and organo-mineral fractions (<53 μm) of the recent + historical biochar and recent biochar, and of field-extracted biochars at micro- and nanoscale. The C functional groups, examined using synchrotron-based soft X-ray (SXR) analyses, from the microaggregates (53-250 μm) were dominated by quinones (284.1 eV), aromatic C (285.2 eV, $1s-\pi^*$ transitions of conjugated C=C), and aliphatic C (287.3 eV) (Fig. 4a). For the mineral-protected fractions (<53 μm), two prominent features were the low intensity of quinones and high intensity of aliphatic C (Fig. 4a). The dominant peaks of aliphatic, amide and carboxylic C (287-289 eV) are the direct consequence of deposition of microbial metabolites or debris, exopolysaccharides, and root exudates onto mineral surfaces^{27,47,48}. These align with the micro-spatial maps produced from the synchrotron-based infrared (IR) microspectroscopy (Fig. 4b). The close correlation between clay minerals and microbial metabolites on the biochar surface supports SOC stabilization, and highlights the importance of clay minerals for the protection of SOC and the control of microbial activity.

The correlation between C forms and clay minerals, confirmed by the IR maps, was further examined on a nanometer scale by directly analyzing the chemical composition of the organo-mineral coatings at the surface and in the pores of field-extracted biochars. Greater intensities of quinones (284.1 eV) and carboxyl C–OOH (288.6 eV) were observed in the 9.5-year aged biochar compared with the 1-year aged biochar (Fig. 5a). A high magnification image of the area where the fungi were located inside a biochar fragment shows a high concentration of irregular pores and a coating of organic material (Figs. 5b & c). Fungi can mine nutrients from minerals by exuding acids^{49,50} which may cause the observed microporosity of organo-mineral-biochar interfaces (Figs. 5b, d, f, h & Fig. S5). Energy dispersive X-ray spectroscopy (EDS) analysis showed that complex changes had occurred on the surface of the biochar over the one-year period (Figs. 5c, 5g). Positively charged nanoparticulate minerals rich in Al, Si, Ca, P, Fe and other cations were attracted to the surface of the negatively charged areas on the biochar. These positively-charged minerals and elements subsequently attracted negatively-charged organic molecules with detectable concentrations of C=C, C–OH, C–N/C=N, C=O, COOH functional groups, quinone bonds and anions thus initiating a process whereby porous clusters are formed on the biochar surface (Figs. 5e, 5i). Similarly, exudates from plants and microorganisms can be deposited around mineral surfaces on the biochar, and cations and minerals can be attracted to these organic molecules. Recent biochar amendment to historical biochar plots would provide unoccupied surfaces and pores in the soil to increase sorption capacity for root exudates⁵¹, which would then serve as binding agents to further enhance aggregate formation⁵². As these clusters are built up, they may also be detached from the biochar either through fluctuating redox conditions and interaction with microbes or perturbation caused by soil invertebrates³⁰.

These biochar micro-sites have a high concentration of free radicals with labile easily-mineralizable organic C and/or inorganics dissolved from the biochar (Table S9). Colloidal biochar particles, leachate, dissolved native OM and rhizodeposits may be further stabilized separately or held together via cation bridging with Ca²⁺, or with Al and Fe oxyhydroxides^{53,54,55} and organo-organic interactions at the nanometer scale⁵⁶ (Figs. 5e, 5i). These processes may be encouraged by oxidation of the biochar

surface as it ages in soil^{36,37}. This is supported by our LC-OCD results where dissolved hydrophobic C fractions and building blocks (medium molecular weight) were greater in the recent + historical biochar compared to the recent biochar (Fig. 5j; Table S10). The analysis of the surface of the 9.5-year aged biochar by C-edge EELS and XPS indicated that most of the oxidized C species were formed in the organo-mineral coating. The concentrations of the different functional groups appear to be influenced by the presence of nanophase Fe, Si and Al oxides⁵⁶ (Fig. 5i).

Global impact of elevating the soil carbon ceiling

Building SOC is a global priority⁹, and our results showed that the SOC storage ceiling can be elevated through single or multiple applications of biochars. We observed a plateau in rhizodeposit accumulation rate over 9.5 years in the historical biochar plots (Fig. 2a; $y = 4.24\ln(x) + 17.6$; $R^2 = 0.95$) which implies that the system was approaching a new (16 % higher) equilibrium for SOC storage, ten years after the initial application. We showed that a strategic application of 10 Mg biochar ha⁻¹ after 8.2 years raised the SOC storage ceiling by a further 2 Mg C ha⁻¹. In summary, this Rhodic Ferralsol under the managed pasture had a C storage capacity of 35 Mg C ha⁻¹ in the surface soil, which increased to 44 Mg C ha⁻¹ one year following the application of biochar, which further increased to 50 Mg C ha⁻¹ after nearly a decade. The C storage ceiling was further raised to 59 Mg C ha⁻¹ where biochar was applied to the historically amended field plots. Of this C storage, 7.6 Mg C ha⁻¹ was attributed to the addition of C from biochar, while 2 Mg C ha⁻¹ was attributed to the stabilization of new C.

The long-term stabilization of rhizodeposits by biochar after 9.5 years has significant implications for elevating the SOC ceiling. Plants release ~50 % of photosynthetically fixed C into the soil, which is available for microbial growth⁵⁷⁻⁵⁹. Global grasslands annually contribute 0.04 Pg C to SOC⁶. The retention and stabilization of belowground C by biochar could play an important part in a natural climate solution for tropical grasslands, which occupy 0.7 Gha of land with an estimated global C content of 30 Pg C. Here we showed a 16 % increase in retention of new C in the recent + historical plots compared with the control via the stabilization of root-derived ¹³C in microaggregates (53-250

202 μm , 11 %) and microbial products and rhizodeposits protected in mineral fractions ($<53 \mu\text{m}$, 5 %) (Fig.
203 3). To demonstrate the potential of raising the C saturation ceiling, we extrapolated this 16 % increase
204 in stabilization based on the global projected biochar production. We estimated the strategic
205 application of biochar can increase SOC storage capacity in Ferralsols by 0.01-0.1 Pg C yr⁻¹ worldwide
206 (Supplementary information). This mechanism would increase the global mitigation potential using
207 biochar as a soil amendment, estimated at 1.3 Pg C yr⁻¹ (centurial average; Woolf et al. 2010), by
208 another 0.8-7.7 %.

209 In our study, we raised the SOC storage capacity in a subtropical pasture with a strategic application
210 of a *Eucalyptus saligna* biochar (550°C) 8.2 years following the original biochar application. Of
211 importance to building soil C stocks, the strategic application of biochar in the aged plots resulted in
212 16 % more new C (*i.e.* microbial products and rhizodeposits) being stored as soil C in both
213 microaggregates (53-250 μm) and mineral fractions ($<53 \mu\text{m}$). Microbial C-use efficiency was improved
214 by lowering the specific enzyme activity and slowing down degradation of SOC and rhizodeposits
215 (negative priming). Our *in situ* spectromicroscopic analyses suggest that the catalytic biochar surfaces
216 accelerated the micro- and nanoscale heterogeneity and temporal variability for new C storage.

217 **Methods**

218 **Field site details**

219 The field experiment was situated at the Wollongbar Primary Industries Institute (28°49'S, 153°23'E,
220 elevation: 140 m), Wollongbar, New South Wales, Australia. The classification and properties of the
221 soil can be found in Weng et al. (2015). Briefly, the Rhodic Ferralsol is fine-textured and Fe-rich mineral
222 soil dominated by kaolinite, gibbsite and goethite. The 100-mm topsoil had a pH_{CaCl2} of 4.5 with total
223 C of 35 g kg⁻¹, total Fe 84 g kg⁻¹, and total Al 67 g kg⁻¹. Details of the initial field site setup in 2006 can
224 be found in Slavich et al. (2013). The treatments (n=3) included (1) *Eucalyptus saligna* biochar
225 incorporated into the topsoil (0-100 mm) at 10 t ha⁻¹ (eq. 1 % w/w, bulk density of 1 g cm⁻³) plus NPK

fertilizer and (2) NPK only (control). The biochar was derived from a single source of above-ground biomass of mature *Eucalyptus saligna* and pyrolyzed at 550 °C with a residence time of 30 mins (Pacific Pyrolysis, NSW, Australia). The physicochemical properties of the biochar can be found in Slavich et al. (2013). A tetraploid annual ryegrass (*Lolium multiflorum*) was broadcast at a seeding rate of 35 kg ha⁻¹ and repeated annually. Urea was applied at 46 kg N ha⁻¹ on six occasions (276 kg ha⁻¹ in total) between winter and spring each year following manual cuts of the pasture to simulate grazing. Basal nutrients were applied annually at sowing (Slavich et al. 2013).

In April 2014, the control (NPK only) and 8.2-year-old biochar (*Eucalyptus saligna*) plots were superimposed with nine subplots (0.5 m × 0.5 m) (Weng et al. 2017). Four treatments were: (1) Recent biochar to historical biochar plots (biochar applied to a part of the historical biochar plots 8.2 years after the trial was established; “recent + historical”); (2) Recent biochar amendment (biochar applied to a part of the control plots 8.2 years after the trial was established, “recent”); (3) Historical biochar amendment (biochar applied 8.2 years previously, “historical”); and (4) Control (nil biochar plots). There was one subplot per field replicate and a total of three field replicates. The biochar added to the control and aged plots was taken from the biochar ‘batch’ applied in 2006, which had been air-dried and archived in sealed 200 L steel containers at room temperature. The details of the subplot set up and installation of belowground respiration collars can be found in Weng et al. (2015). Bulk density of the biochar was 0.332 g cm⁻³ measured using a method described in Quin et al. (2014). The weight of soil-biochar mixture in the topsoil (100-mm) was determined based on the bulk density assessed in each treatment. Before application, the biochar was sieved to <2 mm. The soil/biochar mixture was carefully packed into the subplots. The control subplots were also excavated and repacked to a bulk density of 1 g cm⁻³. A root signature sand collar (50 mm diameter) was installed in each of the control subplots to measure the $\delta^{13}\text{C}$ signature of root respiration. It was packed with acid-washed sand and planted with ryegrass (*i.e.* a down-sized version of the soil plus root respiration collar). Similarly, a biochar+root signature sand collar was packed with a biochar-sand mixture (1 % w/w) in each of the recent + historical biochar subplots. To maintain the root growth into the collars,

NPK fertilizers were applied at the same dose as in Slavich et al. (2013). This study employed the same pasture management regime as Slavich et al. (2013), in terms of ryegrass sowing, NPK applications and weed control.

Periodic ^{13}C pulse labelling to quantify SOC mineralization and root respiration

To understand how plant-biochar-soil interactions affect SOC priming, $^{13}\text{CO}_2$ pulse labelling campaigns were conducted on three occasions: 12 June 2014, 01 August 2014 and 30 July 2015. The procedure of the pulse labelling experiment and a detailed description of quantification of SOC mineralization and root respiration using three-pool C partitioning can be found in Weng et al. (2015, 2018).

Soil sampling was carried out at 8.9, 9.2 and 9.5 years following the first biochar application in 2006. Intact soil cores (40 mm in diameter) were sampled to 80 mm depth within each subplot but outside the respiration collar area to reduce disturbance. The sampled areas were avoided in the ensuing sampling events. The sample was mixed evenly and analyzed for pH, total soil organic C, and microbial biomass carbon (MBC). Soil pH was measured on the samples prepared for the enzyme assay (1:5 w/w ratio in distilled water with constant stirring using a vortex) using an IntelliCAL PHC101 pH probe on a Hach HQ40d portable meter (Loveland, Colorado, USA). The analytical procedures for SOC and MBC can be found in Weng et al. 2015. The remaining soil was stored at -20°C .

At the 15-month soil sampling event, fresh soil was also taken from the soil respiration collars (*i.e.* unplanted) and soil+root respiration collars (*i.e.* planted) to quantify the effect of plant-biochar-soil interactions on catabolic enzyme activity, substrate-induced respiration, and MBC. The MBC was analyzed using the chloroform fumigation method (Van Zwieten et al. 2010). Metabolic quotient of total C or rhizodeposits was then quantified as the ratio of respiration (native SOC or ^{13}C -labelled root respiration) over the total MBC. The metabolic quotient has been used as an indicator of C-use efficiency (Fang et al., 2018). Detailed calculations for determining rhizodeposit-derived respiration are given in Weng et al. (2015).

SOC priming in the plant-biochar-soil systems

The rhizosphere priming of native SOC from the biochar-plant-soil interactions was quantified using a three-pool C partitioning model. Specialized respiration collars were used to isolate soil plus root respiration from shoot respiration^{41,42}. Native SOC mineralization was separated using the $\delta^{13}\text{C}$ signature of biochar plus root (biochar plus root sand collars, Supplementary Information) and total respiration (biochar plus soil plus root collars) after pulse labelling. Moisture content was maintained between 60-80 % field capacity in the root collars to minimize potential C isotopic fractionation during photosynthesis caused by water stress⁴⁴. The $\delta^{13}\text{C}$ signatures of extracted field-aged biochar and fresh biochar (the same biochar archived in a sealed container for 8.2 years) were both $-25 \pm 0.1 \text{ ‰}$. Any interactive effect of biochar and root on the $\delta^{13}\text{C}$ signature of soil would be surpassed by a greater level of $\delta^{13}\text{C}$ enrichment of the root component compared with any isotopic signature contribution from soil and biochar to the $\delta^{13}\text{C}$ signature of the total respiration. A sensitivity analysis of C source partitioning was performed to assess the impact of plant-biochar (C_3 -dominated)-soil interactions on $\delta^{13}\text{C}$ signatures of soil (a mixture of C_3 and C_4 pools). Errors generated from isotopic partitioning were propagated using the first order Tyler series approximations of the variances of native SOC mineralization.

The recovery of ^{13}C in various SOC pools at time t (*i.e.* $A^{13}\text{C}_{i, t}$, in %) was calculated by dividing the amount of ^{13}C (g m^{-2}) in a specific C pool (*i.e.* C_i) by the initial amount of total added $^{13}\text{CO}_2$ (g m^{-2}) at each labelling event (*i.e.* $^{13}\text{C}_{\text{added}}$):

$$A^{13}\text{C}_{i, t} = (^{13}\text{C}_{\text{excess}, t} \times \text{C}_i) / ^{13}\text{C}_{\text{added}} \times 100$$

where i represents soil plus root respiration, root biomass, soil aggregates or its associated fractions, $^{13}\text{C}_{\text{excess}, t}$ indicates the increment of the ^{13}C atom % of an individual C pool from its natural abundance level at a specific sampling time, t .

299 The mineralization of native SOC (C_S) was calculated using the ^{13}C signature of biochar+root ($^{13}\text{C}_{\text{B+R}}$,
300 sand collar) from the plant-biochar-soil systems after pulse labelling:

$$301 \quad C_S(\%) = 100 * (\delta^{13}\text{C}_T - \delta^{13}\text{C}_{\text{B+R}}) / (\delta^{13}\text{C}_S - \delta^{13}\text{C}_{\text{B+R}}) \quad (1)$$

302 where $\delta^{13}\text{C}_T$: $\delta^{13}\text{C}$ signature of the total respiration from the planted system after pulse labelling;
303 $\delta^{13}\text{C}_S$: $\delta^{13}\text{C}$ signature of the soil-derived $\text{CO}_2\text{-C}$ evolved from the unplanted control soil without pulse
304 labelling.

305 The percentage of soil-derived $\text{CO}_2\text{-C}$ in the total respiration from the planted control soil ($C_S(\%)$)
306 was determined (Weng et al. 2015):

$$307 \quad C_S(\%) = 100 * (\delta^{13}\text{C}_T - \delta^{13}\text{C}_R) / (\delta^{13}\text{C}_S - \delta^{13}\text{C}_R) \quad (2)$$

308 where $\delta^{13}\text{C}_T$: $\delta^{13}\text{C}$ signature of the total respiration from the planted control; $\delta^{13}\text{C}_S$: the $\delta^{13}\text{C}$
309 signature of the unplanted control soil; $\delta^{13}\text{C}_R$: the $\delta^{13}\text{C}$ signature of roots, which was determined
310 from root respiration from the root sand collar as described in Weng et al. (2017).

311 Similarly, the percentage of soil-derived $\text{CO}_2\text{-C}$ in the total respiration from the unplanted biochar-
312 amended soil ($C_{S'}(\%)$) was determined:

$$313 \quad C_{S'}(\%) = 100 * (\delta^{13}\text{C}_{T'} - \delta^{13}\text{C}_B) / (\delta^{13}\text{C}_S - \delta^{13}\text{C}_B) \quad (3)$$

314 where $\delta^{13}\text{C}_{T'}$: the $\delta^{13}\text{C}$ signature of the total respiration from the unplanted biochar soil. $\delta^{13}\text{C}_S$: the
315 $\delta^{13}\text{C}$ signature of the unplanted control soil; $\delta^{13}\text{C}_B$: the $\delta^{13}\text{C}$ signature of either fresh (-25.02 ± 0.13
316 ‰) or aged biochar (-25.04 ± 0.11 ‰). Biochars were recovered by hand from field soil samples,
317 thoroughly rinsed with distilled water on a 100 μm sieve and oven-dried at 50°C for 24 h.

318 Rhizosphere priming was calculated in two systems:

319 i. Unamended system (Planted vs. Unplanted)

320 - SOC_{planted, unamended}: soil C mineralization in the planted control calculated by ¹³C-enriched
321 root end-member

322 - SOC_{unplanted, unamended}: soil C mineralization in the unplanted control

323 Rhizosphere priming in the control soil:

324
$$\Delta\text{SOC}_{\text{unamended}} = (\text{SOC}_{\text{planted, unamended}}) - (\text{SOC}_{\text{unplanted, unamended}})$$

325 ii. Biochar-amended system (Planted vs. Unplanted)

326 - SOC_{planted, amended}: soil C mineralization in the planted biochar soil partitioned from ¹³C-
327 enriched 'Biochar+Root' end-member

328 - SOC_{unplanted, amended}: soil C mineralization in the unplanted biochar soil partitioned from
329 biochar end members

330 Rhizosphere priming in the biochar system:

331
$$\Delta\text{SOC}_{\text{amended}} = (\text{SOC}_{\text{planted, amended}}) - (\text{SOC}_{\text{unplanted, amended}})$$

332 SOC priming was the difference in native SOC mineralization between the biochar-amended and
333 control soils:

334
$$\Delta\text{SOC} = (C_s(\%) * C_{T\text{planted}} - C_s'(\%) * C_{T\text{unplanted}}) / 100 \quad (4)$$

335 where $C_{T\text{planted}}$ and $C_{T\text{unplanted}}$ are the total respiration in planted and unplanted systems either with
336 biochar amendment or the control.

337 **Calculated ¹³C atom % (%):**

338
$$^{13}\text{C atom \%} = [(\delta^{13}\text{C} + 1000) * R_{\text{PDB}}] * 100 / [(\delta^{13}\text{C} + 1000) * R_{\text{PDB}} + 1] \quad (5)$$

339 where $R_{\text{PDB}} = 0.01118$.

Sensitivity analysis of isotopic partitioning

Because of the uncertainty of the direction of biochar-induced priming of soil carbon and/or rhizodeposits, the contribution of biochar on the ^{13}C endmember of ($\delta^{13}\text{C}_s$) was assessed. Therefore, three alternative scenarios of three-pool C partitioning were evaluated:

1) dominant positive priming of new C from the C_3 pasture, where $\delta^{13}\text{C}_{s'} = -27\text{‰}$ (*i.e.* the upper boundary, grey dashed line, Fig. 2b);

2) equal native SOC priming and rhizosphere priming, hence, the same ^{13}C signatures of soil+root in the biochar and control plots, where $\delta^{13}\text{C}_{s'} = \delta^{13}\text{C}_s$ (*i.e.* solid lines in Fig. 2b);

3) dominant positive priming of the native C_4 -dominant SOC, where $\delta^{13}\text{C}_{s+R'} = -13\text{‰}$ (*i.e.* the lower boundary, grey dashed line, Fig. 2b).

The boundary conditions were calculated from the published ^{13}C signatures for Scenarios 1 and 3 (Farquhar et al. 1989). The 95 % confidence intervals were the combination of the lowest and highest scenarios ($n = 3$). First order Tyler series of the variances of the percentage of soil respiration, $\text{C}_s(\text{‰})$, were approximated to propagate errors from isotopic partitioning (Derrien et al. 2014).

$$\sigma^2 \text{C}_s(\text{‰}) = (\sigma^2 \delta^{13}\text{C}_T - \sigma^2 \delta^{13}\text{C}_s) / (\delta^{13}\text{C}_T - \delta^{13}\text{C}_s)^2 \quad (6)$$

Enzyme activity and substrate-induced respiration

The determination of catabolic enzyme activities using a soil suspension method is described in Weng et al. (2017). Six treatments were derived from the control, the recent + historical biochar and recent biochar plots in both the unplanted (*i.e.* soil respiration collar) and planted (*i.e.* soil+ root respiration collar) systems (Table S5, Weng et al., 2017). After 7-d incubation at 40 % water-holding capacity (WHC), the activities of four C-degrading enzymes: β -glucosidase, xylosidase, cellulase, and N-acetylglucosaminidase, in the soils were analysed using a fluorescent microplate reader (BMG labtech FLUOstar Omega). Specific C enzyme activity was obtained by dividing the activity of individual

enzymes over the total MBC at each sampling time. These ratios provided an indication of the C- turnover efficiency of the soil microbial community (Kaiser et al., 2015). Substrate-induced respiration was used to measure Community level physiological profiles using the MicroResp™ method (Campbell et al., 2003) with minor modifications. Fresh soil samples, packed in 96-deepwell plates (around 0.5 g per well), were prepared in the same manner as the enzyme experiment (*i.e.* incubation conditions). Each treatment per field replicate was sub-replicated eight times for measurement. The experimental protocol is detailed in Weng et al. (2017). Fifteen C substrates (Table S6) were selected to represent a broad range of soil and root exudates (Campbell et al. 2003; Chapman et al. 2007).

Aggregate size and density fractionation

Aggregate size (dry sieving) and density fractionation was conducted based on the method described by Weng et al. (2018). No large macroaggregates (> 2000 µm) was found in this study. Macroaggregates (250-2000 µm) and microaggregates (< 250 µm) were fractioned into free POM (F-POM, $\rho < 1.6 \text{ kg m}^{-3}$), occluded POM (O-POM, > 53 µm), and mineral-protected soil organic matter (M-SOM, combining silt- and clay-bound SOM, <53 µm).

Belowground ¹³C pools

The C and N content, and $\delta^{13}\text{C}$ signatures of bulk soil, aggregates and fractions were measured using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) (Weng et al., 2015).

3D-FIB-SEM-EDS

The serial section and EDS Mapping of the soil particle was prepared in a FEI SCIOS focused ion beam/scanning electron microscope (FIB/SEM) DualBeam system. The SCIOS FIB/SEM DualBeam system has a vertical mounted SEM column and an ion column sitting at an angle of 52 degrees with respect to the electron column. The particle was located with the aid of the electron beam. Before

any milling, one micrometre thick platinum layer was deposited on the sample surface covering the area of interest to prevent it from damage caused by the ion bombardment in the following steps. The smooth finish of the Pt layer would also help to reduce the curtaining effect during the following milling procedure. The serial sectioning of the volume was carried out at 3nA and 30kV ion beam current and the EDS mapping were collected at 5kV and 6.4nA electron beam current. The voxel size of the SEM images is 84 nm(x) × 84 nm(y) × 1000 nm (z, slicing thickness).

Synchrotron soft X-ray

Synchrotron-based soft X-ray (SXR) analysis was performed at the SXR Spectroscopy beamline (14ID) at the Australian Synchrotron on the microaggregate (53-250 µm) and mineral fractions (<53 µm) from 1) recent biochar-amended plots, and 2) the historically biochar-amended plots; and then biochar recovered from the soil, that is: i) 1-year (aged) and ii) 9.5-year (aged) biochar. The samples were ground to fine powder and mounted on double sided carbon tape affixed to a stainless steel ruler.

The SXR spectra were collected at an angle of 100° to the beam over a photon energy range of 275-325 eV with a step size of 0.1 eV. The energy was calibrated using a graphite standard in the beamline which was collected simultaneously with the I_0 and sample SXR spectra. The double normalization and a pre- and post-edge linear subtraction (background) were conducted using the Athena software (Stöhr 2013).

Synchrotron IR

For infrared microspectroscopy, approximately ~30 free water-stable microaggregates (53–250 µm) and mineral fractions (<53 µm) were hand-picked on a glass fibre filter paper and humidified gently over 18 hours (Lehmann et al. 2017; Hernandez-Soriano et al. 2018). The aggregates and fractions were frozen at –20 °C before being cryo-ultramicrotomed at 200 nm using a diamond knife. No embedding media was used. The multiple sections per sample (n >2) were directly collected on CaF₂ windows (IR transparent).

The sections on CaF_2 were directly scanned at the IR beamline at the Australian Synchrotron using a Bruker Hyperion 3000 infrared microscope and a V80v Fourier transform infrared spectrometer. The detail of the microscope was described in Hernandez-Soriano et al. (2018). The spectral maps were produced in transmission mode from 64 scans with a resolution of 4 cm^{-1} , step size of $5\text{ }\mu\text{m}$. Multiple maps were acquired for each treatment to represent the heterogeneity of the sample.

Maps were processed using the software OPUS 8.2 (Bruker Optik GmbH, Germany), targeting absorbance at 3630 cm^{-1} (O–H groups of clays), 2920 cm^{-1} (aliphatic-C), 1600 cm^{-1} (aromatic-C), and 1035 cm^{-1} (polysaccharides-C)(Hernandez-Soriano et al. 2018). The area of these four absorbance peaks was integrated to the map. A linear regression was conducted to assess the correlation between clay content and the selected C functional groups.

STEM-EDS-EELS and XPS

Forty biochar particles were extracted from the soil samples per plot and were examined using a Zeiss Sigma Scanning electron microscope. Detailed analysis of 5 particles was carried using a Bruker X-ray Dispersive analyser (EDS). A Cs-corrected FEI Titan 80/300 scanning transmission electron microscope (STEM) working at 80 keV, equipped with a Gatan imaging filter Tridiem and an EDX analyzer was utilised to determine the structure and composition of the organo-mineral clusters that had formed on the surface of the aged biochar. Twenty biochar particles were sonicated in ethanol and then a sample of this was placed on a lacey carbon grind as described by Archanjo et al (2017). Detailed examination of 2 clusters was carried out using energy electron loss spectroscopy (EELS) and EDS. X-ray photoelectron spectroscopy (XPS) examination of both whole and crushed ($< 0.5\text{ mm}$) 1-year aged particles of biochar was undertaken. Carbon 1s photoelectron peak was decomposed in five components: C1-C5 (Table S9). The first one (C1) centered in 284.6 eV, typical of electrons in carbon sp^2 bounds (C=C), *i.e.*, delocalized sp^2 electrons. For this component, an asymmetrical line shape was used to fit. The asymmetry of the C1 component, known as a “defect peak”, is related to the localized sp^2 electrons. Electrons of C-C or C-H bounds typically appear with a binding energy shift of 0.9 eV in

relation to sp^2 delocalized electrons, causing a broadening in the first component. The component C2, centered in 286.2 ± 0.2 eV may be attributed to C-OH (phenol or hydroxyl groups), ether (C-O-C) or pyrrolic groups (C-N). Some authors also attribute this component to Csp^3 free radicals. The component C3 is attributed to carbonyl groups (C=O) centered in 287.5 ± 0.4 eV, the component C4 is attributed to the carboxyl groups (COOH) centered in 289.1 ± 0.3 eV, and the last one, the component C5 in 291.4 eV is attributed to the shake-up satellite peak, characteristic of $\pi \rightarrow \pi^*$ transition of electrons delocalized sp^2 .

The concentration of DOC and its fractions, measured by LC-OCD

Dissolved organic carbon (DOC) in a water solution was analysed using liquid chromatography – organic carbon detection (LC-OCD). Two major fractions were: chromatographable organic carbon (CDOC) and hydrophobic organic carbon (HOC). CDOC (hydrophilic fraction) can be categorized into five fractions as a factor of retention time and molecular weight: i) biopolymers, ii) persistent C-like substances, iii) building blocks, iv) low molecular weight acids and v) low molecular weight neutrals. Samples were extracted in distilled water with a ratio of 1:10 (w/v). The solutions were regularly stirred at 50 °C for 24 hours before filtration to differentiate solid and liquid phases.

Calculation and statistical analysis

The cumulative SOC, biochar-C mineralization, and root respiration over 466 d were calculated as the area of a linear interpolation across all measurement points. All statistical analyses were conducted within the R environment (R development core team 2012). When significant F-tests were obtained ($P= 0.05$), means were separated using a least significant difference (LSD) test at the 0.05 probability.

Calculations of global implication for increasing soil carbon sink

Projection for wood biochar production is estimated at 4.8 - 8.3 Pg, based on the total annual production of 5.5 to 9.5 Pg biochar by 2100 (Lehmann et al. 2011) with 87 % of the feedstock as

wood (Jirka and Tomlinson 2013). Using the same application rate in this current study (10 t ha^{-1}), all wood biochar is assumed to be applied to 0.5 - 0.8 Gha in 2100, accounting for up to 100 % of tropical Ferralsol and 36 % of tropical grasslands (Lal 2004). The global C sequestration rate in grasslands is reported between 1.3×10^{-10} and $7.6 \times 10^{-10} \text{ Pg C ha}^{-1} \text{ yr}^{-1}$ (Minasny et al. 2017). The range of C sequestration in grasslands before biochar amendment in 2100 would be around 0.07-0.61 Pg C (*i.e.* 0.5 or 0.8 Gha at 1.3×10^{-10} and $7.6 \times 10^{-10} \text{ Pg C ha}^{-1} \text{ yr}^{-1}$). We found a 16 % increase in retention of new C in the recent + historical plots compared with the control. This would lead to an additional soil C sink potential of 0.01-0.1 Pg C (*i.e.* 16 % of 0.07 or 0.61 Pg C).

Figure Captions

Fig. 1 Conceptual diagram of the formation of organo-mineral coatings on catalytic biochar surfaces over time in a Rhodic Ferralsol. Biochar can act as a bio-catalyst to accelerate formation of organo-mineral microaggregates (53-250 μm) and mineral-protected soil organic matter (<53 μm) on its surfaces and induce negative priming of soil organic carbon. Microbes, fungal hyphae and root hairs can further mine minerals within pores via exudation and dissolution. Microbial necromass covered with minerals is then incorporated into the organo-mineral (<250 μm) and organo-organic (< 100 nm) interfaces. Following wetting-drying and plant growth cycles, organo mineral and organo-organic aggregates break-off from organic matter because of weak bonding. Once these aggregates break off, new mineral layers can form.

Fig. 2 Belowground carbon dynamics in the longest continuous biochar field experiment. a, Changes in total soil organic carbon (SOC, Mg C ha^{-1}) in the control and biochar-amended soils over 9.5 years ($n=3$, $\text{LSD} = 1.1$). Total SOC was measured in the 0-100 soil layer on an equivalent mass basis using Dumas combustion. **b,** Rhizosphere priming as difference in cumulative SOC mineralization between planted and unplanted “recent” biochar amended soil or soil with the “recent + historical” biochar. “Recent” biochar is biochar applied to a part of the control plots 8.2 years after the trial was established (closed triangles). The “recent + historical” amendment is biochar applied to a part of the historical biochar plots 8.2 years after the trial was established (open triangles). Confidence intervals (95%) of “recent” biochar and “recent + historical” biochar amendments are plotted in dashed lines and normalized against the mean squares across all treatments at each sampling event ($n=3$). For biochar amendment, the CI was based on a sensitivity analysis (Online Method Section), which considers the extreme scenarios of contrasting SOC pools (C3 vs. C4 dominated) by differences in $\delta^{13}\text{C}$ signatures. The six arrows represent nitrogen fertilizer additions.

Fig. 3 Allocation and retention of rhizodeposits (^{13}C -enriched) and three-dimensional elemental distribution in a biochar-amended Ferralsol at 9.5 years. a, 8.2 years after the first application, the biochar-amended soil received a recent dose of biochar at 10 Mg ha^{-1} to the historical plots (“recent + historical”). The same total amount ($190 \text{ mg } ^{13}\text{C m}^{-2}$) was supplied in each treatment plot ($n=3$). **b,** Recent biochar (“recent”) was mixed in top 100 mm of soil at 10 Mg ha^{-1} one year before measurement. **c,** 3D FIB-SEM-EDS of an intact soil aggregate ($30 \mu\text{m} \times 25 \mu\text{m} \times 24 \mu\text{m}$) from the “recent + historical” biochar plots. **d,** 3D FIB-SEM-EDS of an intact soil aggregate ($30 \mu\text{m} \times 20 \mu\text{m} \times$

30 μm) from the “recent” biochar plots. Soil sampling was conducted before and 15 days after labelling. The total recovery of ^{13}C labelling is given, including soil + root respiration, root biomass, free- and occluded particulate matter and mineral fractions.

Fig. 4 Synchrotron-based spectromicroscopic analysis of microaggregates (53-250 μm) and mineral fractions (<53 μm) in the unamended control and historical biochar-amended plots with recent biochar addition. **a**, Average SXR spectra of microaggregates (53-250 μm) and mineral fractions (<53 μm) with the “recent + historical” and “recent” biochar amendments ($n=9$, $\text{CV}\% < 3\%$). **b**, Semi-thin (200 nm) sections of free water-stable microaggregates (53-250 μm) and mineral fractions (<53 μm) isolated from the Ferralsol with the “recent + historical” and “recent” biochar amendments analysed using synchrotron-based IR-microspectroscopy. Spectral maps showing the distribution of polysaccharide-C (1035 cm^{-1}), aromatic-C (1600 cm^{-1}), aliphatic-C (2920 cm^{-1}), and mineral-OH (3650 cm^{-1}) were obtained from 64 co-added scans (4 cm^{-1} resolution), lateral resolution 5 μm (bars: 50 μm). The signal intensity for each molecular group varied according to the colour scale shown. The images on the left are optical micrographs of the semi-thin sections.

Fig 5. *In situ* spectromicroscopic analysis of the organo-mineral coating on biochar surfaces and pores over time. **a**, Average SXR spectra of field-extracted “recent” (1-yr aged) and “historical” (9.5-yr aged) biochars ($n=9$, $\text{CV}\% < 3\%$). **b**, high magnification secondary electron image of a pore where fungi exist. **c**, EDS spectrum of the area in **b**. **d**, STEM-HAADF image of organo-mineral clusters on the “recent” biochar surface; **e**, its EELS spectra. **f**, High resolution image of the surface of the organomineral layer inside the pore of the “historical” biochar. **g**, EDS spectrum of the area in **f**. **h**, HAADF image of a deposit attached to the surface of the “historical” biochar. **i**, its EELS spectra. **j**, DOC of bulk soils from the “recent + historical” and “recent” biochar amendments analysed with LCOCD. The hydrophilic fraction is further sub-divided into five categories, i: biopolymer, ii: persistent C, iii: building blocks, iv: low molecular weight acids and v: low molecular weight neutrals.

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687 ZW drafted and wrote the manuscript, experimental design, set-up and conducted experiments, and
688 data collection and analysis; LVZ, BPS and LMM wrote the manuscript, aided in experimental design,
689 critical revision of the article; SJ, ET, BSA and MTR collected and analyzed data, critical revision of the
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Figures

Figure 1

Conceptual diagram of the formation of organo-mineral coatings on catalytic biochar surfaces over time in a Rhodic Ferralsol. Biochar can act as a bio-catalyst to accelerate formation of organo-mineral microaggregates (53-250 μm) and mineral-protected soil organic matter (<53 μm) on its surfaces and induce negative priming of soil organic carbon. Microbes, fungal hyphae and root hairs can further mine minerals within pores via exudation and dissolution. Microbial necromass covered with minerals is then incorporated into the organo-mineral (<250 μm) and organo-organic (< 100 nm) interfaces. Following wetting-drying and plant growth cycles, organo mineral and organo-organic aggregates break-off from organic matter because of weak bonding. Once these aggregates break off, new mineral layers can form.

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