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Root penetration in deep soil layers stimulates mineralization of millennia - old organic carbon

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Keywords

Deep soil carbon, Rhizosphere priming effect, ^{14}C dating, ^{13}C labelling, Microbial community structure ,Microbial energy limitation, Soil C persistence

Abstract

Climate and land-use changes modify plant rooting depth, signifying that organic matter with long residence times in deep soil layers can be exposed to rhizospheres and associated microbial activities. The presence of roots in soils stimulates mineralization of native soil C, via a process termed the rhizosphere priming effect (RPE), which may in consequence lead to loss of soil C. By growing a deep rooting grass, *Festuca arundinacea*, on soil columns and under continuous dual labelling (^{13}C- & ^{14}C-CO_2), we show that root penetration up to 80 cm into a soil profile stimulated mineralization of ~15,000 year-old soil C. The RPE, after normalization with root biomass, was similar along the soil profile indicating that deep C is as vulnerable to priming as surface C. The RPE was strongly correlated with respiration of plant-derived C, and a PLFA marker representative of saprophytic fungi (18:2ω6c) across all soil layers. Moreover, experimental disruption of soil structure further stimulated soil C mineralization. These findings suggest that the slow soil C mineralization in deep layers results from an impoverishment of energy-rich plant C for microorganisms (especially for saprophytic fungi), combined with a physical disconnection between soil C and microorganisms. Based on our results, we anticipate higher mineralization rates of deep millennia-old SOM in response to deeper root penetration which could be induced by changes in agricultural practices and climate.

1.Introduction

During the last decade, the organic carbon stored in deep (> 20 cm depth) soil layers has received increased attention of the scientists concerned with soil feedbacks to climate change as well as the mitigation of global warming (Fontaine et al., 2007; Rumpel and Kögel- Knabner, 2011; Marin-Spiotta et al., 2014; Medlyn et al., 2015; Kaneez-e-Batool et al., 2016). More than 50% of the 2344 Gt C, stored as organic C in soils, is located below 20 cm (Batjes, 1996; Jobbágy and Jackson, 2000). This large pool of deep C is capable of massively altering the global C cycle and climate if its mineralization by soil microorganisms is stimulated in response to global changes. Moreover, 14C dating of soil C has shown that the deep C is often thousands of years old whereas the surface C turns over in decades (Torn et al., 1997; Trumbore, 2000; Jenkinson et al., 2008). This finding has led to many investigations to understand the origin of deep C persistence and to define the conditions of a quasi-permanent storage of carbon in soils (Fontaine et al., 2007; Salomé et al., 2010). The applied perspective of these investigations relates to developing technologies to mitigate

the rising atmospheric CO_2 and consequent global warming by sequestering C in stable compartments. For example, it has been proposed to use and breed plant species with deep roots to fix atmospheric CO_2 and sequester organic carbon in deep soil layers (Hurd, 1974; Lorenz and Lal, 2005; Carter and Gregorich, 2010; Iversen, 2010; Kell, 2011).

The presence of roots in soils stimulates microbial mineralization of native soil C, a process termed the rhizosphere priming effect (RPE) (Kuzyakov et al., 2000; Dijkstra and Cheng, 2007; Cheng et al., 2014). Several processes can contribute to the RPE but the most frequently suggested are co-metabolism and N mining (Fontaine et al., 2003; Kuzyakov, 2010; Allison et al., 2014; Chen et al., 2014). In co-metabolism, the enzymes released by microorganisms during decomposition of plant C (plant litter and rhizodeposits) accidentally interact with native soil organic C accelerating its mineralization. According to mining theory, microorganisms growing on plant C, secrete enzymes decomposing soil organic matter to acquire nutrients. More recently, Keiluweit et al. (2015) suggested that some root exudates can also stimulate carbon loss by liberating organic compounds associated with minerals. The RPE is a major determinant of SOC turnover in surface soils. Increasing the rate of SOC mineralization by up to 400% (Finzi et al., 2015; Shahzad et al., 2015), the RPE can lead to a negative soil C balance in certain conditions (e.g. N poor soils), that is, C input to soil may decrease soil C content (Fontaine et al., 2004a; Dijkstra and Cheng, 2007). Deep C is less exposed to RPE than the surface soil C since most plant roots are concentrated in the surface layer (Fontaine et al., 2007). However, climate and land use changes such as the use of deep ploughing and drought-resistant deep-rooting crop species are modifying the plant rooting depths (Lorenz and Lal, 2005; Schenk and Jackson, 2005; Kell, 2011; Lorenz et al., 2011). Some previously protected deep soil layers are exposed to plant rhizosphere and its cohort of microbial activities possibly leading to RPE. However, the effect of plant rhizosphere on mineralization of deep soil C is unknown.

Diverse mechanisms have been proposed to explain the persistence of deep soil C. Historically, deep C has been viewed as functionally inert presumably because of its protection from microbial decomposition through organo-mineral associations or its existence as recalcitrant chemical structures (Torn et al., 1997; von Lützow et al., 2006). However, resistant chemical structures can persist at decadal timescales only (Kleber et al., 2011). The increasing importance of organo-mineral associations, where organic compounds are associated with reactive mineral phases, in stabilizing SOC down the profile has been demonstrated for a range of soils (Torn et al., 1997; Rumpel and Kögel-Knabner, 2011). However, for certain soils organo-mineral associations do not explain deep soil C persistence (Fontaine et al., 2007). Ewing et al. (2006) proposed that lack of

physical disturbance in deep layers retards aggregates from breaking thereby promoting the persistence of deep C since the aggregates deny microorganisms the access to C substrates. Moreover, because most soil C compounds are located in pores inaccessible to microorganisms, it has been proposed that conservation of soil structure induces a spatial disconnection between soil C and microbes thereby slowing down mineralization process (Salomé et al., 2010; Schimel et al., 2011; Dungait et al., 2012). For the sake of simplicity, spatial disconnection and protection of soil C by aggregates will be referred to as "physical protection" in this study. Fontaine et al. (2007) attributed the persistence of deep C to a lack of easily decomposable organic compounds owing to limited roots that supply soil microbes with essential sources of energy (rhizodeposits, litter etc.) in top soil layers, a process named "energy limitation". In their soil incubation, the mineralization of millennia-old deep C could only operate in the presence of cellulose suggesting that the old C was not an energetically profitable source of C for microorganisms. Different studies, while discussing the origin of deep C persistence, have often considered the processes of physical protection and energy limitation antagonistic at least unrelated (Salomé et al., 2010; Schmidt et al., 2011; Dungait et al., 2012).

The main objective of this study was to determine whether root penetration and exudation in deep soil layers can stimulate mineralization of millennia-old organic carbon, that is, induce a RPE. Moreover, to clarify the processes controlling the persistence of deep soil C, we tested whether energy limitation and physical protection mechanisms together can explain this persistence. The experimental approach consisted of three phases. First, a deep rooting plant species, *Festuca arundinacea*, was sown on undisturbed soil columns after re- moving the upper 0–10 cm soil layer to favour a deeper root penetration in soil. The grass was grown for 511 days under an atmosphere containing dual-labelled CO2 (13C & 14C) allowing for the quantification of overall plant activity and the quantification of the RPE. During the second phase, soil columns were sliced in three independent soil layers (surface, intermediate and deep layers) that were subsequently incubated for 7 days. The RPE and ^{14}C age of primed soil C were determined for each soil layer during this incubation. During the third and last phase, soil structure of each layer was disrupted followed by incubation of these disrupted soils. The release of CO_2 induced by soil disturbance was quantified over 79 days.

2.Materials & methods

2.1.Soil sampling

Soil was sampled from a temperate upland grassland located in the environmental research observatory (SOERE) established by the French National Institute for Agricultural Research (INRA) in central France in 2003 (Theix, 45°43′N, 03°01′E). The local climate is semi-continental, with a mean annual temperature of 9 °C and an average annual rainfall of 760 mm. The site has been under grassland for more than 60 years. The soil is a drained Cambisol of 1 m depth. It is a silty, isohumic soil developed from volcano-granitic colluviums with little developed horizons. Moreover, the soil is hydromorphic with moderate permeability in surface. In March 2009, ten intact soil columns of ~9.8 cm diameter were taken within 1 m distance to each other from 0-80 cm depth. The upper 0–10 cm were removed before inserting the core in the PVC tube to remove the existing plants and a large proportion of their fresh litter, which would have confounded the effect of roots' presence on soil C. A percussion core drill equipped with a steel tube that can be opened from sideways was used to extract the soil columns when the soil was relatively wet (soil moisture ~ 30% dry weight; water potential ~ −100 kPa). This method allowed to recuperate entire soil columns and transfer them in PVC tubes (80 cm long, 9.8 cm internal diameter) while preserving the structure of soil columns. The plant experiment was established on these intact soil columns representing 10–80 cm of the soil profile. Two of the ten sampled columns were not transferred in PVC tubes and were cut horizontally in 10–33 cm, 33–56 cm and 56–80 cm layers for determining initial pH, soil SOC contents and isotopic composition ($^{13}C/^{12}C$ ratio). A more detailed description of soil properties and chemical composition of SOC along soil profile of the study site can be found in (Fontaine et al., 2007).

2.2.Experiment

Soil moisture at field capacity

All tubes containing the soil columns were irrigated until the soil columns were water saturated. The soil tubes were then weighed after 48 h of water percolation to determine soil moisture at field capacity. This estimation was used to keep the soil moisture between 75 and 100% of soil field capacity throughout the experiment using an auto- mated drip irrigation method.

Plant establishment and labelling

The experiment was established in the fields of INRA, Clermont Ferrand, France. Four of these tubes containing intact soil columns were sown with *Festuca arundinacea* Schreb. at a density of 2000 seeds m^{-2} and four were kept bare as controls. *F. arundinacea* has one of the deepest roots among plant species commonly found in temperate permanent grasslands (Picon-Cochard et al., 2011; Pagès and Picon-Cochard, 2014). Its roots can easily go to 80 cm of soil depth with 95% of roots staying in upper 63 cm (Fan et al., 2016). It should be noted that the total length of the soil columns in our study was 70 cm (i.e. 10–80 cm). Immediately after germination, all planted and bare soil tubes were placed in a greenhouse supplied with a continuous flow of air containing dual labelled CO_2 ($^{13}CO_2$ & $^{14}CO_2$). A schematic diagram of the labelling system and picture of the mesocosm containing the planted and unplanted pots is shown in Fig. S1. A screw compressor, a self-regenerating adsorption dryer capable of generating decarbonized air at a rate of up to 5000 standard liters per minute and residual CO_2 below 25 µmol CO_2 mol^{-1} air, an air reservoir, gas cylinders containing fossil fuel derived CO_2 and a humidifier (1 m^3:1 m^2 cross corrugated cellulose pads) constitute the main parts of the labeling system. Ambient air was taken into the system by a compressor, its CO_2 and H_2O contents and all other particles were scrubbed by a molecular sieve. This decarbonized air was then mixed with fossil-originated CO_2 which is naturally depleted in ^{13}C and ^{14}C. A mass flow meter served to control the CO_2 injection rate. Before entering the mesocosm, the la- belled air mixture was passed through a humidifier where the air passed through corrugated sheets damped by continuously falling water. Flow rate of the whole system was controlled by pressure regulators. During daytime, around 30% of decarbonized dry air was diverted to one of the two molecular sieves in order to regenerate it. Therein, regeneration of molecular sieve was done every six minutes. There was no labeling during night time. The concentration of CO_2 in the mesocosm was maintained at 400 ± 20 ppm. The air in mesocosm was renewed al- most twice a minute to avoid the uptake of soil-derived (unlabelled) CO_2 by plants and limit temperature increase due the greenhouse effect. The temperature increase can reach +2 °C in some specific conditions (for instance, a sunny day during winter) but was not significant when averaged for the whole year. Indeed, the air production system cooled down the air during the process. The relative humidity within the mesocosm was maintained around 50–60%. The CO_2 used for labelling was obtained from a single lot (Supplier: Air Liquide) so that the isotopic composition of CO_2 remained identical throughout the experiment. The delta ^{13}C and the ^{14}C content of CO_2 were equal to -38.55 ± 0.07‰ and 0% of modern carbon, respectively. These experimental conditions were maintained during 511 days; a duration long enough for the grass to extensively colonize deep soil layers of soil columns.

Plant management

Plants were fertilized with ammonium-nitrate after 20, 122 and 364 days following sowing (equivalent of 70 kg N ha^{-1}, 70 kg N ha^{-1}, 60 kg N ha^{-1}, respectively). Plants were also fertilized with phosphorus, potassium, sulphur and magnesium after 20 days following sowing (equivalent of 100 kg P_2O_5 ha^{-1}, 200 kg K_2O ha^{-1}, 20 kg S ha^{-1} and 60 kg MgO ha^{-1}, respectively). The fertilizer doses were based on the regional practices in low mountain grasslands in Massif Central (Pontes et al., 2015). Bare soil columns were kept unfertilized to avoid an accumulation of mineral nutrients which may lead to lower respiration rates and over-estimation of the RPE (Fontaine et al., 2004a). To avoid the accumulation of mineral nutrients, planted and bare soil columns were further leached with water monthly. The plants were clipped thrice to 5 cm from ground 122, 189 and 413 days after sowing as per the growth of the plants.

2.3.Respiration measurements

A detailed schematic diagram of the experimental approach particularly the respiration measurements, has been provided in Fig. 1. The experimental approach consisted of three phases.

Phase 1: intact soil columns with plants

Soil respiration was measured using a method previously described by us (Shahzad et al., 2012). Briefly, on the eve of each respiration measurement, the tubes were irrigated to field capacity ensuring that all the respiration measurements were done in similar soil moisture conditions. The next day, tubes were taken out of the greenhouse and sealed in air-tight PVC chambers (height 100 cm, diameter 15 cm) for 24 h. Absence of light stopped photosynthesis consequently stopping the plant absorption of soil-respired CO_2. The CO_2 released by soil-plant system was trapped in a soda lime trap (100 ml 1M NaOH) placed in the respiration chambers. By analysing the gas samples taken from inside the respiration chambers on chromatography (Agilant 3000 micro GC), we found that the CO2 remaining in chamber after trapping never exceeded 1% of the CO_2 trapped in soda lime. This result signified that the trapping was efficient and the isotope fractionation during trapping had negligible effect on isotopic composition of trapped CO_2. However, by maintaining very low CO_2 concentrations in chambers (few ppm CO_2) the soda lime can stimulate the diffusion of soil CO_2 decreasing the mean CO_2 concentration in soil pores. This might result in an over-estimation of soil respiration activity irrespective of treatments (planted and bare soils). The

respiration from intact soil columns was measured after 119, 138, 185, 187, 200, 230, 285, 348, 377, 409 and 460 d after plant germination depending on intensity of plant-soil activities.

Phase 2: incubation of surface, intermediate and deep soil layers

After 511 days, plants were clipped to base. Each planted and bare soil tube was then sliced horizontally into three sections with a thin hacksaw. From top to bottom, they represented surface (10–33 cm), middle (33–56 cm) and deep (56–80 cm) soil layers. During the cutting process, the soil structure in these three layers was maintained thanks to the PVC tube and sufficient soil moisture. There must have been minor disturbance in soil structure on the cross section of soil columns through which the hacksaw passed. However, we consider them 'intact' soil layers because almost all the soil volume escaped the disturbance in soil structure. These intact soil layers in PVC tubes were placed in respiration chambers (height 100 cm, diameter 15 cm) for seven days. The soil CO_2 was trapped as previously described except that the soda lime trap was 200 ml 1.5M NaOH.

Phase 3: incubation of disrupted soils

Soils were taken out of the PVC tubes, their structure was broken and each replicate was thoroughly mixed with hands in a similar way. A 10 g of soil sample from each soil layer were incubated in 500 ml flasks at 20 °C and a water potential of -100 kPa for 79 days. Before incubation, all the visible roots were removed from the soils. The CO_2 released by soils was trapped in 10 ml 1M NaOH placed inside flasks. The soda traps were replaced after 8, 21 and 51 days of incubation. The flasks were flushed with CO_2-free air during each soda trap replacement. The effect of soil disruption on soil C mineralization was determined by comparing total soil CO_2 emissions before and after breakdown of soil structure.

Analysis of soda traps

The CO_2-C trapped was quantified by analysing the dissolved in- organic carbon content of soda lime using an automated analyzer (TNM-L Shimadzu). To analyse the isotopic composition ($^{13}C/^{12}C$ and $^{14}C/^{12}C$ ratios) of trapped CO_2, a precipitate of $BaCO_3$ was produced by adding $BaCl_2$ in excess in soda lime. The $BaCO_3$ was collected by filtration, rinsed and dried at 60 °C. The $^{13}C/^{12}C$ ratio of $BaCO_3$ was determined with an elemental analyzer coupled to an Isotope-Ratio Mass Spectrometer (IRMS). Given the cost of ^{14}C analysis, only the $BaCO_3$ obtained from incubation of intact upper (10–33 cm) and deepest soil layer (56–80 cm) were analysed for $^{14}C/^{12}C$ ratio. The $^{14}C/^{12}C$ ratio was measured at Poznan Radiocarbon Laboratory in Poland by accelerator

mass spectrometry as described in details on (Poznan Radiocarbon Laboratory, ul. Rubież 46, 61612 Poznan, Poland). The CO2 trapped from disrupted soils was not analysed for $^{13}C/^{12}C$ ratio.

2.4.Root & soil microbial biomass & PLFA measurements

Roots

Soil samples were sieved at 2 mm. Roots retained by sieve and all visible roots in sieved soil were handpicked and washed with tap water. The collected roots were oven dried at 60 °C for 48 h and weighed.

Microbial biomass

Microbial biomass was measured using a modified (Fontaine et al., 2011) fumigation-extraction method (Vance et al., 1987). Briefly 10 g of soil was extracted with 40 mL of 30 mM K_2SO_4 after shaking for 1 h. Another 10 g of soil sample was fumigated with alcohol free chloroform under vacuum conditions in a glass desiccator for 24 h. Chloroform was removed from the soil by ventilation and soils were extracted with 40 mL of 30 mM K_2SO_4. The extracts were filtered (0.45 µm) and then lyophilized. The recovered crystals were analysed for C content and $^{13}C/^{12}C$ ratio with an elemental analyzer coupled to an IRMS. Total microbial biomass (MBt, mg C kg^{-1} dry soil) was calculated as:

$$MB_t = \frac{C_f - C_{nf}}{k} \quad (1)$$

where C_f and C_{nf} were the carbon content of crystals obtained from extraction of fumigated and non-fumigated soil samples, respectively, and k was extraction yield of microbial biomass (k=16%, Fontaine et al., 2004b).

A 2 g subsample from each sample was sieved at 2 mm and freeze- dried. Phospholipid fatty acids (PLFAs) were extracted using a modified method of Bligh & Dyer (1959) (Frostegard et al., 1991). After fatty acid methyl esters (FAMEs) were obtained (Dowling et al., 1986), they were analysed by GC/MS (4000 GC/MS, Varian). Full details can be seen in Methods S1 and Shahzad et al. (2015).

2.5.Isotope partitioning of soil C and plant C in respiration and microbial biomass

Respiration

The respiration of unlabelled soil C (R_s, mg CO_2-C kg^{-1} dry soil day^{-1}) and labelled plant C, coming from root and mycorrhizal respiration and microbial mineralization of labelled plant materials (R_p, mg CO_2-C kg^{-1} dry soil day^{-1}) were separated using the classical mass balance equations:

$$R_t = R_p + R_s \quad (2)$$

$$R_t \times {}^{13}F_t = R_p \times {}^{13}F_p + R_s \times {}^{13}F_s \quad (3)$$

where R_t was the total respiration of plant-soil (mg CO_2-C kg^{-1} dry soil day^{-1}) and ${}^{13}F_t$ its fractional abundance, ${}^{13}F_S$ the fractional abundance of soil C and ${}^{13}F_p$ the fractional abundance of plant C. The ${}^{13}F_S$ was determined for the three soil layers by analysing soil C by IRMS. ${}^{13}F_p$ was determined by analysing plant roots (See Methods S2 for details). The two unknowns, R and R, can be determined by solving this system of equations (1) and (2):

$$R_s = R_t \times \frac{{}^{13}F_t - {}^{13}F_p}{{}^{13}F_s - {}^{13}F_p} \text{ ; where } R_p = R_t - R_s \quad (4)$$

Microbial biomass

Similarly, the soil-C derived microbial biomass (MB_s, mg C kg^{-1} dry soil) and plant-C derived microbial biomass (MB_p, mg C kg^{-1} dry soil) were separated as follows:

$$MB_t = MB_p + MB_s \quad (5)$$

$$MB_t \times {}^{13}F_{BMt} = MB_p \times {}^{13}F_p + MB_s \times {}^{13}F_s \quad (6)$$

where MB_t is the total microbial biomass measured by fumigation-extraction and ${}^{13}F_{BMt}$ its fractional abundance. The ${}^{13}F_{BMt}$ was calculated as

$${}^{13}F_{BMt} = \frac{C_f \times {}^{13}F_f - C_{nf} \times {}^{13}F_{nf}}{C_f - C_{nf}} \quad (7)$$

where ${}^{13}F_f$ and ${}^{13}F_{nf}$ are the fractional abundances of fumigated and non-fumigated soil samples respectively.

2.6.Calculation of RPE and ^{14}C age of unlabelled soil carbon released by RPE

The rhizosphere priming effect (RPE, mg CO_2-C kg^{-1} dry soil day^{-1}) induced by the plant was calculated as:

$$RPE = (R_s \text{ planted soil}) - (R_s \text{bare soil}) \quad (8)$$

The $_{14}C$ content of total CO_2 emitted by the plant-soil system (percentage of modern carbon, pMC_t) is determined by the mixture of plant-C respiration (R_p) and soil-C respiration (R_s), each of these two sources of respiration having distinct ^{14}C content. The ^{14}C content of R_s (pMC_s) can be determined using the dual labelling of plant material and following mass balance equation (Fontaine et al., 2007):

$$R_t \times pMC_t = R_s \times pMC_s + R_p \times pMC_p \quad (9)$$

Which gives after transformation:

$$pMC_s = \frac{R_t \times pMC_t - R_p \times pMC_p}{R_s} \quad (10)$$

where pMC_p is the ^{14}C content of plant root which was determined by AMS. The pMC_t was determined by analysing the ^{14}C content of trapped CO_2 ($BaCO_3$). R_t was determined by measuring the total amount of trapped CO_2. R_s and R_p were determined based on the ^{13}C isotopic partitioning (Eq. (4)). Therefore, pMCs is the sole unknown of Eq. (10). After its calculation, pMCs was converted into age before present (year BP) using the conventional Libby half-life of ^{14}C (5730 years).

Finally, the ^{14}C content of carbon released by RPE (pMC_{RPE}) was determined as:

$$pMC_{RPE} = \frac{(R_s \text{ planted soil}) \times pMC_s - (R_s \text{ bare soil}) \times pMC_{\text{bare soil}}}{RPE} \quad (11)$$

where $pMC_{\text{bare soil}}$ is the ^{14}C content of CO_2 emitted from the bare soil. The pMC_{RPE} was converted into age (years BP) as previously described.

2.7.Statistical analyses

We used analysis of variance (ANOVA) to test the effects of plant presence and soil depth on root biomass, total CO_2-C release, soil-de- rived CO_2-C, total microbial biomass and soil-derived

microbial bio- mass. ANOVA was also used to test the effect of soil depth in soil columns under plants on the rhizosphere priming effect (RPE), RPE per unit root biomass and RPE per unit plant-derived microbial biomass. Correlations were determined between root biomass and the RPE, between plant-derived CO_2-C (R_p) respired from three soil layers and the RPE and between the plant-derived C assimilated in microbial biomass (MB_p) and the RPE. Moreover, a two-way ANOVA was used to assess the effect of plant presence and soil depth and the interaction of the two factors on distribution of individual PLFA biomarkers (relative abundance % in total PLFA measured). The correlation between PLFA biomarkers and soil-derived CO_2-C was assessed using Pearson's coefficient of correlation. All statistical tests were performed using Statgraphics Centurion XVI (StatPoint Technologies, Inc, Warrenton, VA, USA).

3.Results

3.1.Soil profile and its colonisation by roots

The soil pH was acidic and slightly increased with depth, with 6.3, 6.5 and 6.6 in 10–33 cm, 33–56 cm and 56–80 cm layers respectively. The SOC content of this isohumic Cambisol slightly decreased with depth (g kg^{-1} soil), from 27.8 ± 0.6 to 22.3 ± 0.6 and 21.5 ± 0.2 g C kg^{-1} in 10–33 cm, 33–56 cm and 56–80 cm layers respectively. After 511 days of plant growth, root biomass was present in all the three layers (Fig. 3d). The root biomass strongly decreased with depth, from 6.1 ± 0.8 g dry matter (DM) kg^{-1} soil in the 10–33 cm layer to 0.88 ± 0.2 and 1.37 ± 0.2 g DM kg^{-1} soil in the 33–56 cm and 56–80 cm layers respectively. After the 511 days of experiment, no roots were found in the bare soil columns across the three depths indicating that roots from previous plants were decomposed.

3.2.Isotopic composition of soil and plant material

The mean $\delta^{13}C$ of SOC for soil profile was −25.96 ± 0.27‰ reflecting the C3 photosynthetic pathway of plant species of the studied temperate grassland. The $\delta^{13}C$ of SOC slightly, but significantly, in- creased with depth, from -26.5 ± 0.1‰ in the 10–33 cm layer to -25.8 ± 0.0‰ and -25.6 ± 0.1‰ in the 33–56 cm and 56–80 cm layers, respectively (P = 0.00). The plant material was successfully dual labelled. The $\delta^{13}C$ of plant root (−55.8 ± 0.2‰) was substantially depleted compared to SOC.

3.3.Respiration fluxes of intact soil columns

Total CO emissions (R_t) were 4 11 times higher in planted soils than in bare soils mainly depending on the season (Fig. 2a). R_t were maximal during the summer and autumn period (July to October) and lowest during winter period (December to March). The R_t included shoot and root respiration, mycorrhizae respiration and microbial mineralization of SOC and plant C. Plant presence significantly increased emissions of unlabelled soil-originated CO_2 (R_s) throughout the duration of the experiment (Fig. 2b). The R_s from planted soil columns ranged between 2.8 ± 0.2 to 13.0 ± 0.2 mg CO_2-C kg^{-1} soil day^{-1}, whereas it ranged between 1.1 ± 0.0 to 4.6 ± 0.4 mg CO_2-C kg^{-1} soil day^{-1} for the bare soil columns. The RPE, when represented as % of the R_s from bare soils, ranged between 113 and 264% across the measurements. The seasonal variation of Rs faithfully follows the one of total CO_2 emissions and plant C mineralization. For all the dates of measurement, RPE averaged 170% of the R_s from bare soil columns.

3.4.Respiration fluxes of intact soil layers

The values of respiration fluxes from the three layers shown in Fig. 3 were obtained after slicing of soil columns and incubation of individual soil layers. In bare soils, the three layers emitted similar amounts of CO_2 ($P > 0.05$). Across the three soil depths, presence of plant roots strongly stimulated total emissions of CO_2-C (R_t) (Fig. 3a). Plant stimulation of R_t was a function of soil depth: under plant presence R_t was multiplied by ∼12 in the 10–33 cm layer and by ∼3 in the 33–56 cm and 56–80 cm layers.

Plant presence also significantly increased R_s in the three studied soil layers (Fig. 3b). The RPE represented an increase of 48–193% relative to bare soil depending on soil depth. The absolute value of RPE strongly decreased with depth, from 8.09 ± 0.3 m CO_2-C kg^{-1} soil day^{-1} in the 10–33 cm layer to 1.86 ± 0.3 mg CO_2-C kg^{-1} soil day^{-1} and 2.23 ± 0.4 mg CO_2-C kg^{-1} soil day^{-1} in the 33–56 and 56–80 c soil layers respectively (Fig. 3c). However, the RPE when expressed per unit root biomass, was similar throughout the soil profile (Fig. 3e).

3.5. ^{14}C dating of SOC-derived CO_2-C

In bare soils, the ^{14}C content of the CO_2 released from the intact soil layers significantly decreased down the soil profile, from 86.8 ± 1.7 pMC in the 10–33 cm soil layer to 80.3 ± 2.0 pMC in the 56–80 cm soil layer (Table 1). The age calculation showed that 1174 (+167/-164) and 1810 (+171/-168) years old C was released from the 10–33 cm and 56–80 cm soil layers respectively.

In planted soils, the ^{14}C content of soil-originated CO_2 was obtained after subtracting the labelled plant-originated C from total CO_2 emissions. The 14C content of soil-originated CO_2 significantly decreased with the plant presence (R_s) (Table 1) indicating that the RPE induced the release of very old aged C. Estimated ages of soil C emitted through RPE were 6004 (+1084/-958) and 15,612 (+1914/-1553) years in the 10–33 cm and 56–80 cm soil layers, respectively (Table 1).

3.6. Plant effect on microbial biomass & communities along soil profile

Plant presence increased the total microbial biomass (MB) by 60% in the surface soil and 40% in the two deep soil layers (Fig. 4a). This increase was due to the formation of new labelled MB since the soil-derived MB did not change with plant presence (Fig. 4b). Plant-derived MB was significantly higher in the upper soil layer than in the deeper ones which contained similar MB (Fig. 4c).

Plant presence significantly stimulated the relative abundance of arbuscular mycorrhizal fungi (16:1ω5c) and certain saprophytic fungi (i.e. 18:2ω6c) and Gram positive bacteria (i.e. i17:0 and i16:0). With depth, the relative abundance of Gram positive (i.e. i17:0, i16:0, i15:0 but not the a15:0) and Gram negative bacteria (i.e. 19:0cy, 17:0cy and 16:1ω9c) significantly increased whereas that of two out of three saprophytic fungal biomarkers (i.e. 18:1ω9c and 18:1ω9t) decreased. The fungal biomarker 18:2ω6c showed no significant variation along the soil profile (Table 2).

3.7. Plant and microbial control of RPE along soil profile

In whole soil columns, a strong positive correlation was found between RPE and R_p, the respiration of labelled plant C, which is a proxy of plant photosynthesis and rhizodeposition (Fig. 5). The relative abundance of a saprophytic fungal group (18:2ω6c), arbuscular mycorrhizae (16:1ω5c) and Gram-positive bacteria (i16:0) were found positively related to soil-derived CO_2-C (R_s), which

included the RPE in planted soils, across plant and soil depth treatments ($P < 0.05$, Table 2). The saprophytic fungi showed the strongest relation with Rs (Pearson $r = 0.87$, $P < 0.001$, Table 2, Fig. S3). The relative abundance of a Gram-positive bacteria represented by i17:0 and Gram-negative bacteria represented by 19:0cy showed a negative relationship with the R_s (Table 2).

3.8.Respiration fluxes of disturbed soil layers

Disruption of soil structure caused a release of large amounts of CO_2, increasing R_t by a factor of 1.8–26.3 depending on soil layers and whether the soil was planted or not before soil disturbance (Fig. 6). The effect of soil disturbance on R_t was higher for bare (4.8–26.3 versus 1.8 to 9.2) and increased with soil depth (Fig. 6). The higher R_t in the disturbed soil persisted over the 79 days of incubation although the difference between disturbed and undisturbed soils decreased significantly over time.

4.Discussion

Vulnerability of millennia-old C to microbial mineralization

Release of millennia-old C triggered by the plantation of a deep rooting plant, *Festuca arundinacea,* is key finding of our study. This result shows that the microbial mineralization of millennia-old deep C does not necessarily require extreme soil disturbance and/or addition of pure C substrate as in lab incubations (Ewing et al., 2006; Fontaine et al., 2007) but it also occurs in a scenario that can happen in natural and agro-ecosystems (deeper penetration of plant roots along soil profile). Moreover, in response to root penetration in deep soil layers, the release of deep C is rapid (< 2 years) and concerns intermediate as well as deeper soil layers. The vulnerability of deep C to microbial mineralization is comparable to surface C since similar RPE/root biomass ratio was found along the soil profile (Fig. 3e).

The difference of ~ -30‰ in $\delta^{13}C$ between plant and soil material is the largest ever reached in studies measuring the RPE induced by living plants. We conducted a sensitivity analysis where we varied the δ13C of SOC by 1‰ from the measured value to account for the possible fractionation during respiration. We found that the relative RPE values remain similar across the three soil depths and the ^{14}C age reduces by 12% in surface and 6% in deep soil layer (Table S1). The main results and conclusions of the study hold true after this analysis. Root biomass contained small quantity of ^{14}C (^{14}C content = 8.77 ± 0.84 pMC, Table 1), which was mainly explained by residual atmospheric

CO_2 in the air flowing out of molecular sieve. However, the ^{14}C content of root biomass remained highly depleted compared to the current ^{14}C content of the atmospheric CO_2. This permitted a robust estimation of 14C content and age of primed soil carbon.

The C released from bare soils was relatively old, with ~1100 and 1800 years old C released from upper (10–33 cm) and the deepest (56–80 cm) layers of bare soils respectively (Table 1). This result can be explained by the fact that the soil columns used in the experiment were taken after the upper 10 cm, where modern organic matter is typically concentrated, were removed. The SOC in the studied soil profile contained very old C as shown in Fontaine et al. (2007) who dated SOC in the 60–80 cm layer and found a mean age of 2007 ± 31 years. Moreover, the incubation of soil layers for ^{14}C dating of CO_2 was done after 511 days of incubation of whole soil columns in greenhouse. This period is sufficient to enable microorganisms to decompose most plant residues thereby releasing the most recent C. With the exhaustion of plant residues, the diet of surviving microbial populations is progressively dominated by millennia-old C (Fontaine et al., 2007).

Impact of plant and soil depth on soil microbial composition and SOC mineralization

Plant presence was the primary factor determining the microbial community composition followed by soil depth (Table 2). Thus, changes in soil physico-chemical conditions induced by plant roots (exudation of C, uptake of nutrients, water etc.) have more profound consequences on soil microbial communities than those induced by soil depth alone (lower oxygen availability and physical disturbance). Moreover, though structure of microbial communities changed along the soil profile, the capacity of rhizospheric microbial communities to mineralize soil C (RPE) remains unchanged along the soil profile as shown by the similar RPE/root biomass ratios (Fig. 3e) Therefore, the turnover of SOC along soil profile is mainly driven by roots and their transformations of soil environment.

Our findings also showed a strong positive correlation between a marker of saprophytic fungi (18:2ω6c) and magnitude of RPE or SOC mineralization (R2 = 0.91, Fig. S3, Table 2). This biomarker is also found in plant roots. However, the contribution of plant roots in this fungal biomarker is negligible (Kaiser et al., 2010) and we carefully removed all visible plant remains in the small soil subsamples used to extract soil PLFA. Therefore, the PLFA 18:2ω6c can be used as an indicator of biomass of saprophytic fungi in our experiment. The co-occurrence of this fungal marker and RPE add to the list of studies (Bell et al., 2003; Fontaine et al., 2011; Shahzad et al., 2015) suggesting that this group of saprophytic fungi has dominant role in RPE even if other microbial communities are involved as well (Nottingham et al., 2009; Pascault et al., 2013). To

induce RPE, microorganisms need to co-metabolize the energy-rich plant C and the nutrient-rich SOC. However, these two sources are mostly spatially separated (Fontaine et al., 2011). Therefore, the predominance of fungi in inducing RPE might be explained by their ability to grow as mycelia which confers them a unique capacity to explore soil space, mine large reserve of SOM and reallocate in mycelia (Frey et al., 2000, 2003) energy and nutrients from different parts of the soil.

Origins of deep soil C persistence

What does determine the persistence of SOC in sub-soils over long timescales? Our study suggests that one of key factors explaining deep SOC persistence is the lack of plant rhizodeposition, an essential source of energy for microbial activities. Although the structure of microbial communities changed with soil depth, the ability of these communities to mineralize SOC remains similar when they are supplied with plant C (Figs. 3e and 5). Increase in abundance of microbial communities in deep soil capable of degrading the added litter at similar rates as their surface counterparts have been reported earlier as well (Sanaullah et al., 2016).

Our findings also show that physical protection of SOC is another important mechanism for the deep SOC persistence (Fig. 6). Disruption in soil structure, which exposes the soil C 'pockets' to microorganisms (Ewing et al., 2006; Salomé et al., 2010), caused large and persistent release of CO_2 across all soil layers (Fig. 6). Although the physical protection seems to exert a control on SOC mineralization in all soil layers, its importance increases with depth as shown by the greater impact of soil disturbance on deep soil layers than surface soil (Fig. 6). The limited contribution of physical protection to SOC persistence in surface soil is explained by the fact that most sources of soil physical disturbance (rainfall, drought-rewetting or freeze-thaw cycles, bioturbation, root penetration) arise from the top. The organo-mineral associations are another important mechanism contributing to persistence of soil C, which is likely more important down the soil profile (Torn et al., 1997; Rasmussen et al., 2005; Rumpel and Kögel-Knabner, 2011).

The dual control of SOC persistence by the physical protection of SOC and the energy limitation of microorganisms, found for our study site, suggest that these two mechanisms work in tandem (Figs. 3 and 6). The physical separation of soil C from its decomposers exacerbates the energy limitation of decomposers because extracellular enzymes from and soluble C to microorganisms have to diffuse over long distance (Fierer and Schimel, 2002; Xiang et al., 2008; Chabbi et al., 2009). While diffusing, part of extracellular enzymes is inactivated (Dungait et al., 2012) decreasing the return on investment of microorganisms. However, the physical separation of soil C and microorganisms is, as shown in our study, only relative since the plant supply of energy-rich substrates to soil fungi

allows them to explore soil with their mycelia and mineralize soil C (Figs. 3 and 6). Similarly, because rhizodeposition could alleviate the physical separation of microorganisms vis-à-vis SOC, the soil disturbance provoked relatively much lower increase in soil C mineralization in planted soils than in control soils (Fig. 6).

Limitations of the study

As with any experimental work, our study has limitations that re- quire further research. First, we tested whether root penetration in deep soil layers can stimulate mineralization of millennia-old organic C in a pot experiment where *Festucal arundinacea* was grown on intact soil columns aftert the upper 10 cm were removed. These experimental conditions were designed to promote root penetration in deep layers. Now the effect of deeper root penetration into soil profile on soil or- ganic turnover and storage should be studied in a more realistic way (e.g. deep ploughing) and by making a full C balance considering the loss of old soil C due to RPE and the formation new soil C due to humification of plant C. Second, deviation in ^{13}C isotopic composition between C sources (SOC or plant C) and released CO_2 cause un- certainties in the quantifications of RPE and calculated ages (Table S1.) However, the robustness of our findings is explained by the specific technology of ^{13}C labelling we developed (Shahzad et al., 2012) in order to maintain a stable δ13C difference of ∼30‰ between plant C and SOC (instead of ∼10–15‰ difference generally used). However, we acknowledge that we tested only one source of isotopic fractionation; therey could be other sources of fractionation as well which need to be researched. Third, as shown previously in these grasslands, the grass clipping reduces the RPE by more than 50% due to reduced plant photosynthesis and thereby lower rhizodeposition (Shahzad et al., 2012). Therefore, we argue that the RPE measured over 7d incubation for intact soil layers after cutting aboveground biomass must have underestimated the RPE. Consistently, over the whole duration of the experiment, the average RPE was 170% of the R_s from bare soils for whole columns (Fig. 2) whereas, after soil columns were sliced, RPE was ∼102% of the R_s from bare soils.

Implications

Our results indicate that the increase in rooting depths in response to climate (Schenk and Jackson, 2005; Heimann and Reichstein, 2008; Kell, 2011) and land-use changes (Lorenz and Lal, 2005; Kell, 2011; Lorenz et al., 2011) may stimulate the mineralization of millennia-old soil organic matter stored in deep soil layers. This stimulation will not necessarily lead to a decrease in deep soil C stock since plant litter also contributes to the formation of new soil C. A carbon balance including the loss of old soil C and the formation of new soil C could not be made in this study because the duration of the experiment was too short to induce significant change in soil C stocks. The largest risk of mineralization of millennia-old C from deep soil concerns the use of deep ploughing and drought-resistant deep-rooting crop species in response to climate changes (Kell, 2011). In sixty years of intensive agriculture, annual crops have depleted most of soil C reserve of surface soils by stimulating the mineralization/humification ratio (Lal, 2004; Perveen et al., 2014). Given that deep C is as vulnerable as surface C to environmental changes (Figs. 3e and 5), the exploitation of deep soil layers by cropping systems might extend the soil C loss to deep soil layers resulting in large CO_2 release. Finally, our findings may help to understand the life strategy of plant species with deep roots. The competitive advantage for plants in investing broad deep root structure is not trivial since the amount of available nutrients in deep soil layers is typically low (Mckinley et al., 2009). However, given that these plant species could stimulate nutrient cycles locked in millennia-old SOM, they might have developed a strategy to escape from the strong plant competition for available nutrients in surface soil.

Author contribution

TS and SF designed and performed the experiment, TS, SF and GA performed analyses, TS and SF wrote the initial draft, all the authors contributed to refine the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2018.06.010.

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Table 1. C content, ^{14}C content (% of modern carbon, pMC) and ^{14}C age (years before present) of soil-derived C respiration by bare soils, planted soils and rhizosphere priming effect during 7 days' incubation of two soil layers i.e. 10–33 cm & 56–80 cm. The incubation was preceded by growth of a deep-rooted grass, *Festuca arundinacea*, for 511 days on whole soil columns (10–80 cm) which were then sliced without disturbing soil structure. The perennial grass was continuously exposed to dual-labelled CO_2 (^{13}C & ^{14}C) allowing the quantification of RPE and the age of released soil C (Fontaine et al., 2007). Values are means (n=4), standard errors are given in parentheses. Standard errors of ^{14}C age are asymmetric due to exponential decay of ^{14}C.

Soil depth	**Soil-derived C respiration(Rs)*** **(mg C kg^{-1} soil)**	**^{14}C Content (pMC)**	**^{14}C Age (Years BP)**
10-33 cm			
Bare soil	29.3 (2.1)	86.8 (1.7) †	1,174 (+167/ -164)
Planted soil	87.8 (2.1)	60.4 (4.5) ‡	4,162 (+637/-591)
Rhizosphere priming effect**	58.5 (1.8)	48.4 (5.9) ‡	6,004 (+1,084/-958)
56-80 cm			
Bare soil	28.1 (1.5)	80.3 (2.0) †	1,810 (+171/-168)
Planted soil	45.3 (2.8)	53.4 (1.6) ‡	5,195 (+206/-201)
Rhizosphere priming effect**	17.2 (2.4)	15.1 (4.4) ‡	15,612 (+1.914/-1,553)

* It was measured over 7 days on the three soil layers after slicing the soil columns on which plants grew for 511 days.

** RPE was calculated as the difference of R_s from planted and bare soils.

† It was determined directly on the carbonates ($BaCO_3$) of the CO_2 trapped in lime after it was released from bare soils after 7 days of incubation.

‡ It was calculated based on the R_s and R_p components of the total respiration using equations 9, 10 and 11

Table 2. The individual PLFA biomarkers (relative percentage of the total PLFA measured) as affected by the presence of plants & soil depth. The last column shows the correlation between a biomarker and soil-derived CO_2-C (R_s, mg CO_2-C kg^{-1} soil) across all the soil depths and plant treatment. Significance is represented by *** when P < 0.001, ** when P < 0.01 and * when P < 0.05. NS, non-significant. 10–33, 33–56 & 56–80 represent the three soil depths (cm). Letters against each depth show level of significance with the letter 'a' representing the highest value.

PLFA	Microbial Group	Effects (LSD significance order)			Pearson r
		Plant	Soil depth	Plant × Soil depth	
18:2ω6c	Saprophytic Fungi	***Planted> Bare	NS	***	0.87***
16:1ω5c	Mycorrhizal Fungi	**Planted > Bare	NS	NS	0.69***
i17:0	Gram Positive Bacteria	** Planted > Bare	*10-33 b, 33-56 a, 56-80 ab	NS	-0.53**
i16:0	Gram Positive Bacteria	**Planted > Bare	*10-33 ab, 33-56 b, 56-80 a	NS	0.44*
19:0cy	Gram Negative Bacteria	NS	*10-33 b, 33-56 a, 56-80 ab	NS	-0.42*
18:1ω9t	Saprophytic Fungi	NS	*10-33 ab, 33-56 a, 56-80 b	NS	-0.37
17:0cy	Gram Negative Bacteria	NS	**10-33 b, 33-56 a, 56-80 c	NS	-0.36
a15:0	Gram Positive Bacteria	NS	NS	NS	0.27
i15:0	Gram Positive Bacteria	NS	**10-33 b, 33-56 b, 56-80 a	NS	0.14
16:1ω9c	Gram Negative Bacteria	NS	***10-33 b, 33-56 b, 56-80 a	*	-0.13
18:1ω9c	Saprophytic Fungi	NS	**10-33 a, 33-56 a, 56-80 b	NS	-0.02

Fig. 1. Schematic diagram of experiment in general and the measures of the respiration from columns, intact soil layers and disrupted soil layers.

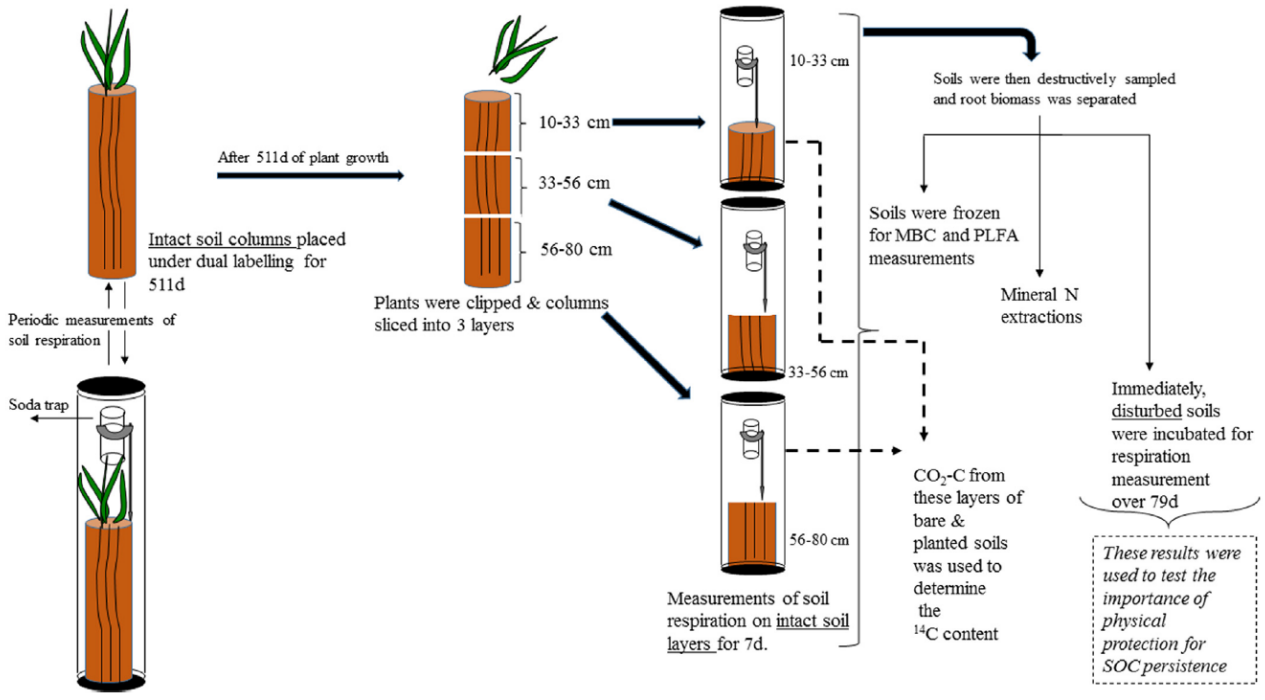

Fig. 2. Mean daily emission rate of total CO_2-C (R_t) (a) and soil-derived CO_2-C (R_s) (b) determined on intact soil columns, bare and planted, during 511 days of experiment. Rhizosphere priming (RPE) was calculated as the difference in R_s between planted soil and bare soil. Vertical lines and arrows along R_t of planted soil in panel (a) represent plant clipping and fertilization treatments respectively. The months on the base of panel (a) are given to indicate the season of the year in northern hemisphere. In panel b, numbers indicate RPE expressed in percentage of R_s from bare soils.

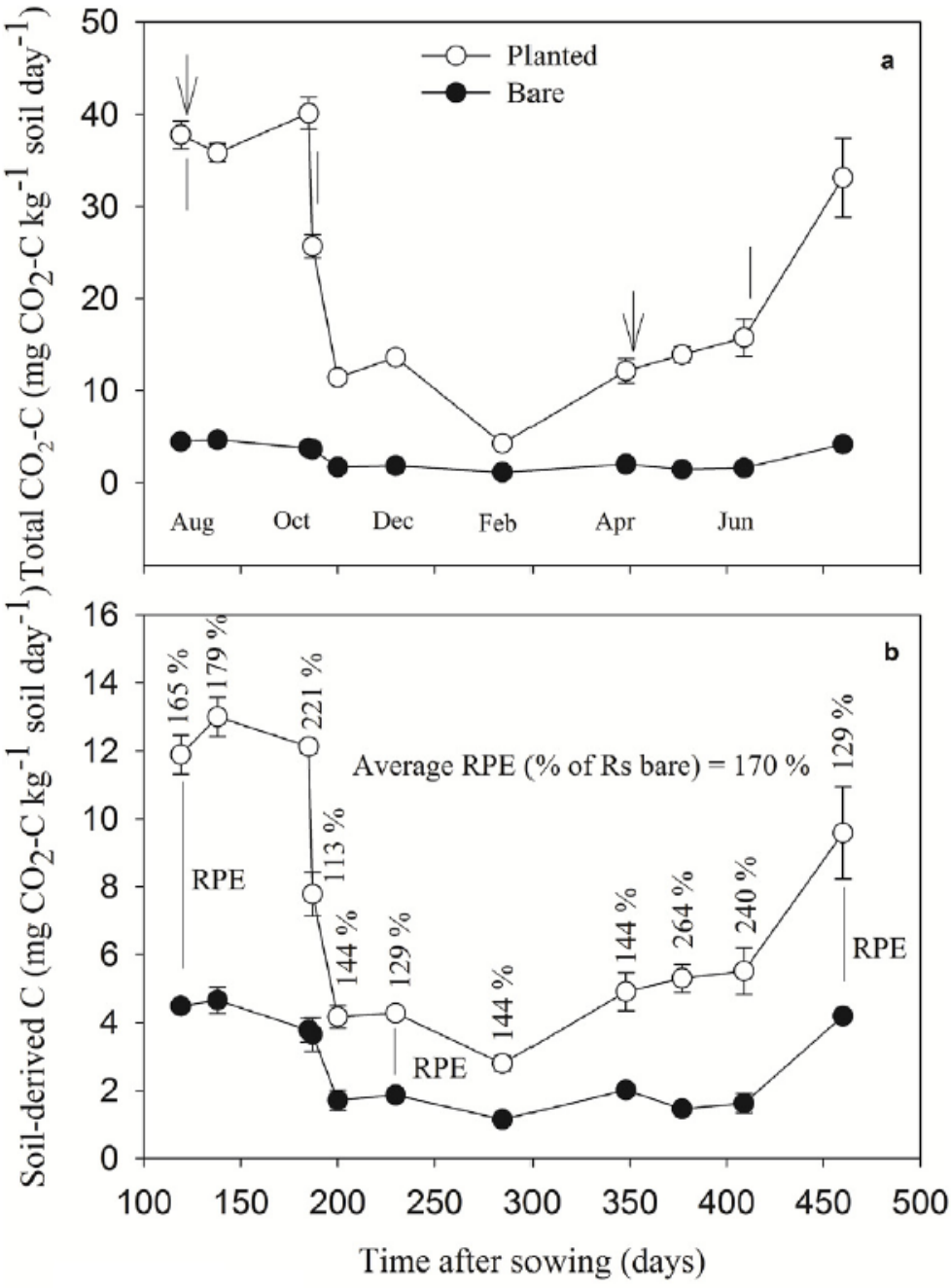

Fig. 3. Effect of a deep rooting grass (Festuca arundinacea) on soil C fluxes in three undisturbed soil layers: Total CO_2-C (R_t) (a), soil-derived CO_2-C (R_s) (b), Rhizosphere priming effect (RPE) (c), Root bio- mass (g/kg soil) recovered from the three soil depths (d), and RPE induced per unit of root biomass (e). Soils layers were 10–33, 33–56 and 56–80 cm. Fluxes were measured during 7 days of incubation after whole soil columns were sliced into three layers while preserving the structure of soil layer.

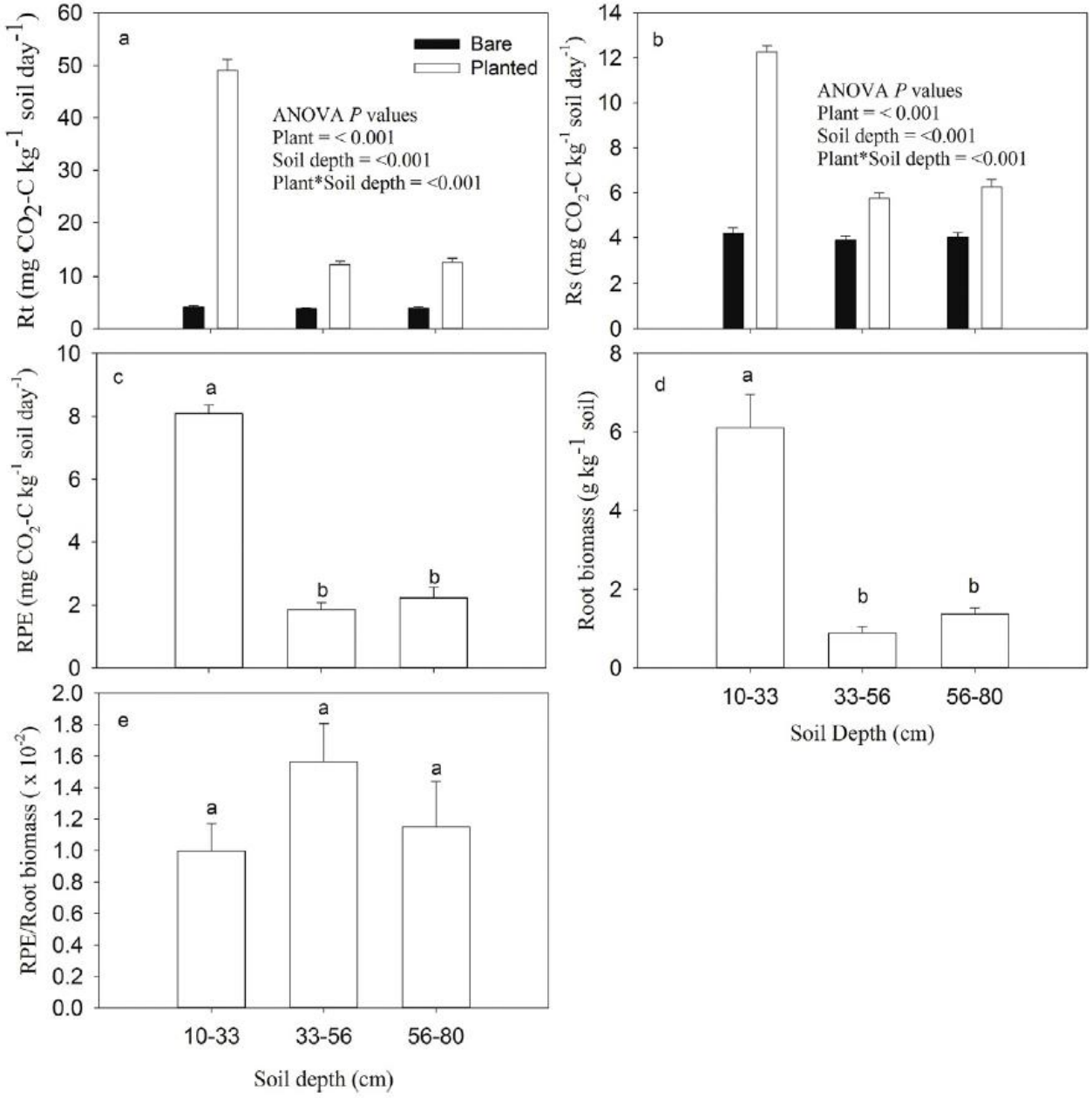

Fig. 4. Effect of a deep rooting grass (*Festuca arundinacea*) on total microbial biomass C (MB_t) (a), soil-derived microbial biomass C (MB_s) (b), plant-derived biomass C (MB_p) (c). These were determined in three soil depths.

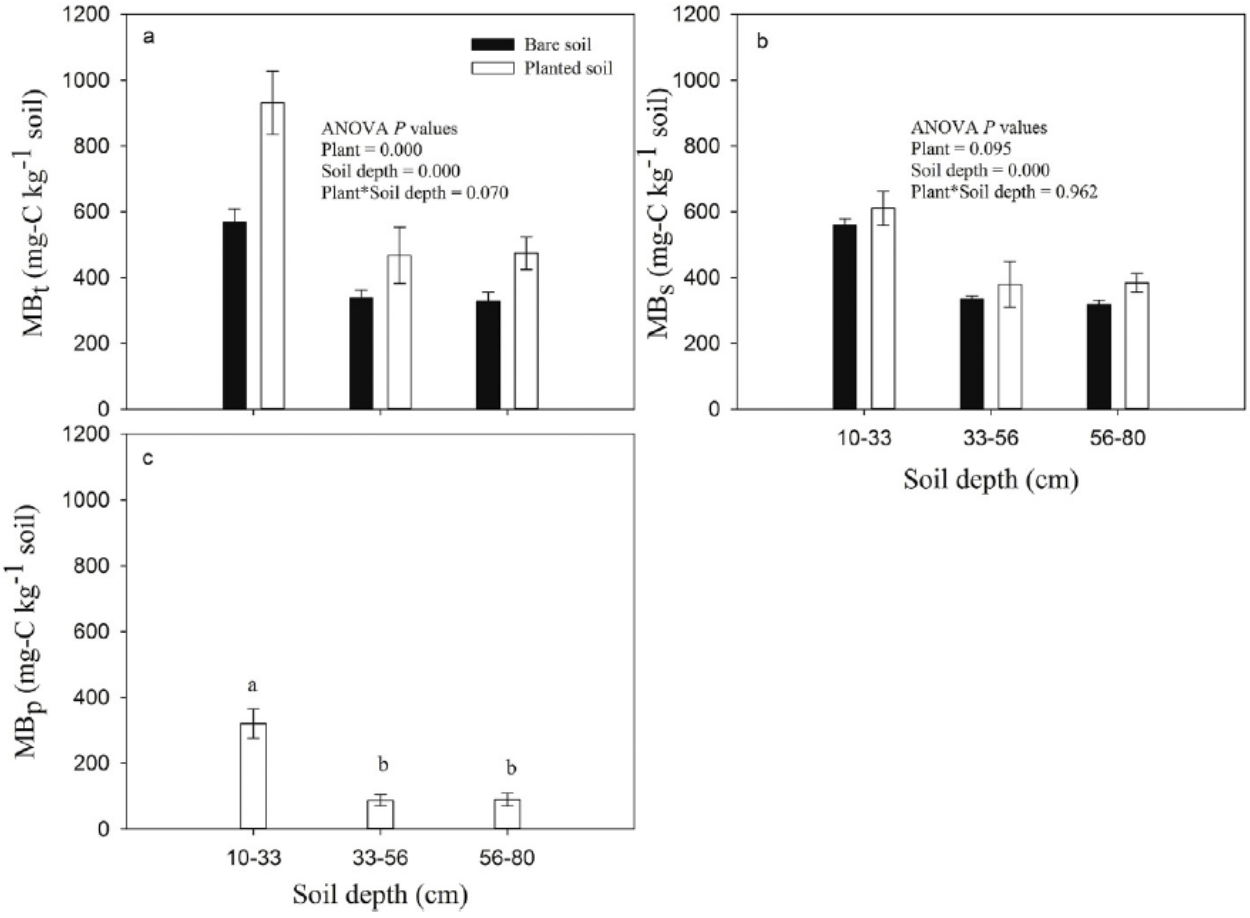

Fig. 5. Relationship between rhizosphere priming effect (RPE) and plant-derived C respiration (R_p) in whole soil columns throughout the experiment.

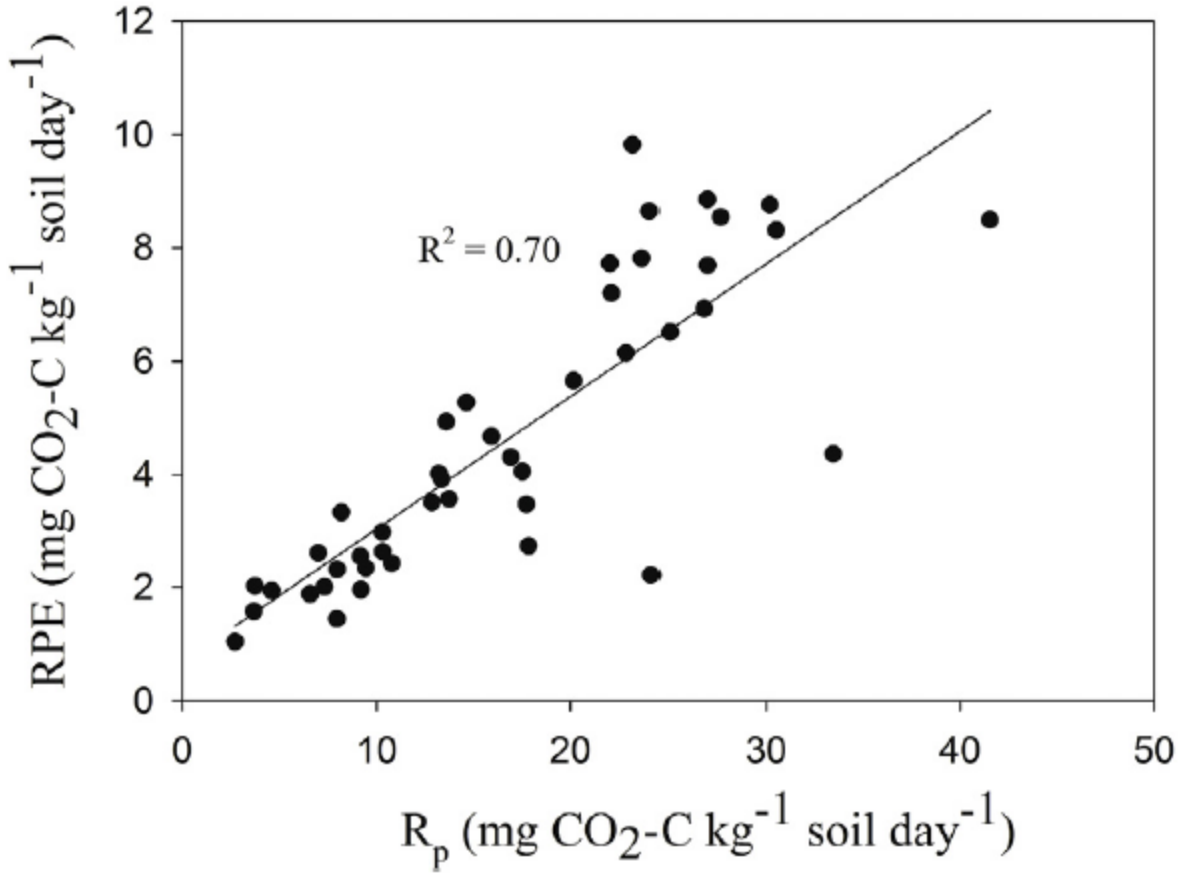
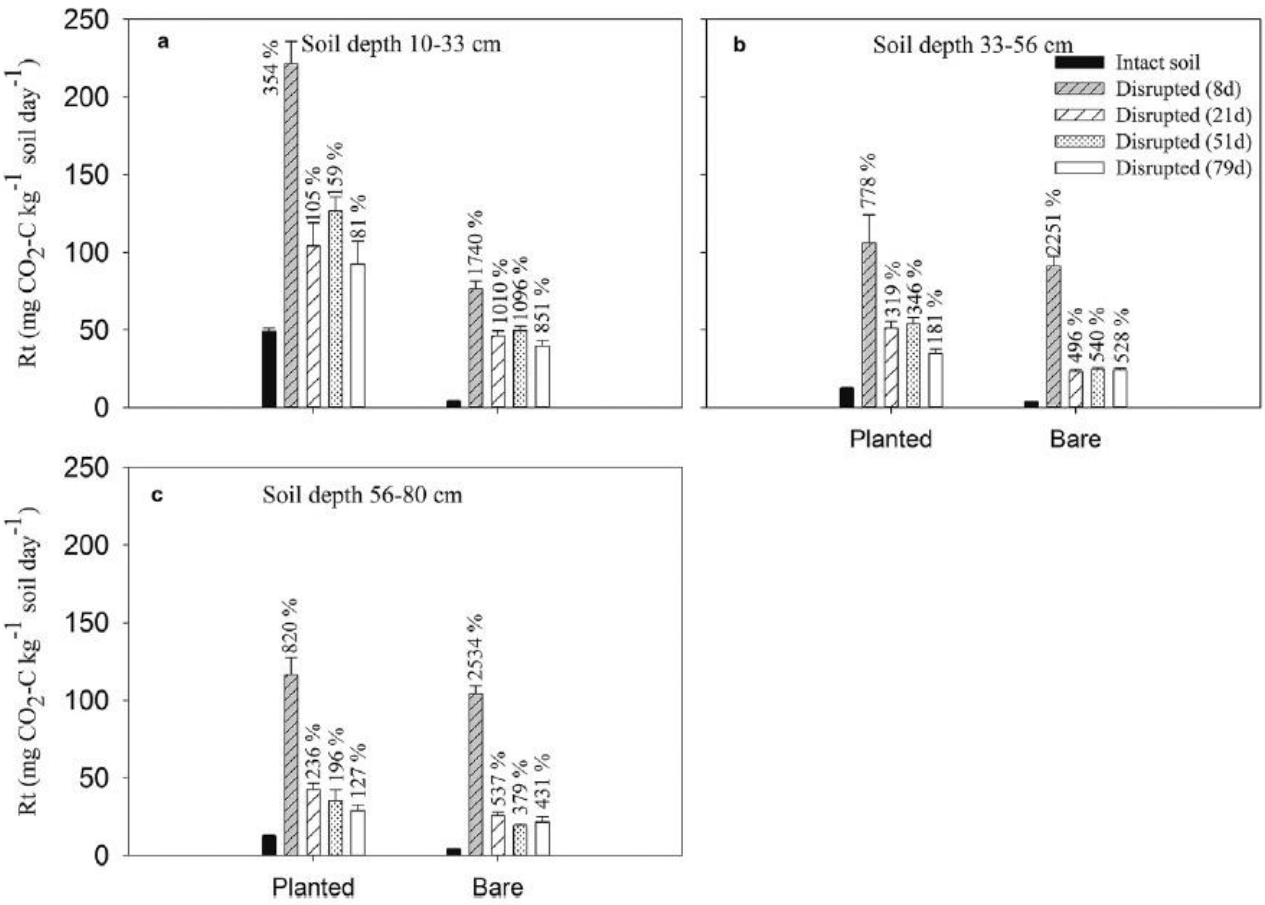
Fig. 6. Effect of soil structure disruption on the total CO_2-C emissions (R_t) from planted and bare soil layers over a period of incubation of 79 days in 10–33 cm (a), 33–56 cm (b) and 56–80 cm (c) soil layers. The R_t measured over 7d before disruption was used as a reference to estimate the effect of soil disturbance on R_t. The numbers above bars represent the percentage of R_t increase induced by soil disruption (average on 4 replicates). Note: Soil disruption was done once at the start of incubation. Within brackets in the legend of the graph are mentioned the days after the initial soil disruption.