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# **Contribution of exudates, arbuscular mycorrhizal fungi and litter depositions to the rhizosphere priming effect induced by grassland species**

Tanvir Shahzad<sup>1,2</sup>, Claire Chenu<sup>3</sup>, Patricia Genet<sup>4,5</sup>, Sébastien Barot<sup>6</sup>, Nazia Perveen<sup>1</sup>, Christian Mougin<sup>7</sup>, Sébastien Fontaine<sup>1</sup>

<sup>1</sup> INRA, UR 874 Unité de Recherche sur l'Ecosystème prairial, 5, Chemin de Beaulieu 63039 Clermont Ferrand cedex 2, France.

<sup>2</sup> Department of Environmental Sciences, Government College University Faisalabad, Allama Iqbal Road, 38000 Faisalabad, Pakistan

<sup>3</sup> AgroParisTech, UMR BioEMCo (Univ. Paris 6, Univ. Paris 12, AgroParisTech, ENS, CNRS, INRA, IRD), 78850, Thiverval-Grignon, France

<sup>4</sup> UPMC Paris 6 – CNRS UMR 7618, 46 rue d'Ulm, 75230, Paris Cedex 05, France

<sup>5</sup> Université Paris Diderot – Paris 7, 75205, Paris Cedex 13, France

<sup>6</sup> IRD-Bioemco, UMR 7618, Ecole Normale Supérieure, 46 Rue d'Ulm, F-75230 Paris Cedex

<sup>7</sup> INRA UR 251 PESSAC, Centre de Versailles-Grignon RD 10- 78026 Versailles Cedex

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## Abstract

The presence of plants induces strong accelerations in soil organic matter (SOM) mineralization by stimulating soil microbial activity - a phenomenon known as the rhizosphere priming effect (RPE). The RPE could be induced by several mechanisms including root exudates, arbuscular mycorrhizal fungi (AMF) and root litter. However the contribution of each of these to rhizosphere priming is unknown due to the complexity involved in studying rhizospheric processes. In order to determine the role of each of these mechanisms, we incubated soils enclosed in nylon meshes that were permeable to exudates, or exudates & AMF or exudates, AMF and roots under three grassland plant species grown on sand. Plants were continuously labeled with  $^{13}\text{C}$  depleted  $\text{CO}_2$  that allowed distinguishing plant-derived  $\text{CO}_2$  from soil-derived  $\text{CO}_2$ . We show that root exudation was the main way by which plants induced RPE (58-96% of total RPE) followed by root litter. AMF did not contribute to rhizosphere priming under the two species that were significantly colonized by them i.e. *Poa trivialis* and *Trifolium repens*. Root exudates and root litter differed with respect to their mechanism of inducing RPE. Exudates induced RPE without increasing microbial biomass whereas root litter increased microbial biomass and raised the RPE mediating saprophytic fungi. The RPE efficiency (RPE/unit plant-C assimilated into microbes) was 3-7 times higher for exudates than for root litter. This efficiency of exudates is explained by a microbial allocation of fresh carbon to mineralization activity rather than to growth. These results suggest that root exudation is the main way by which plants stimulated mineralization of soil organic matter. Moreover, the plants through their exudates not only provide energy to soil microorganisms but also seem to control the way the energy is used in order to maximize soil organic matter mineralization and drive their own nutrient supply.

## **1. Introduction**

Rhizosphere processes contribute almost half of the total CO<sub>2</sub> emissions from the terrestrial ecosystems to the atmosphere at global level (Schimel, 1995). They are also suggested to play a significant role in mediating ecosystem feedbacks to climate change through their effects on net primary productivity, organic matter decomposition, nutrient cycling and carbon (C) storage (Grayston et al., 1997; Kaiser et al., 2010; Cheng et al., 2013). This has led to studies to better understand the magnitude, controls and direction of rhizosphere processes over soil organic matter (SOM) dynamics (Dijkstra et al., 2006a,b; Dijkstra and Cheng, 2007; Cheng et al., 2013; Dijkstra et al., 2013; Drake et al., 2013). Despite this increased interest, there are still many uncertainties regarding which processes strongly accelerate SOM turnover in the rhizosphere thereby making the predictions of soils' feedback to climate change contradictory (Davidson and Janssens, 2006).

Rhizodeposition, i.e. the release of root exudates, mucilage and sloughed-off root border cells, constitute significant inputs of labile carbon into soil (Paterson, 2003; Nguyen, 2009). Overall, up to 20% of the net C fixed by plants is released into soil in the form of rhizodeposition during vegetative period (Hütsch et al., 2002). The main component of rhizodeposition is root exudates through which 10-100 times more carbon is released than mucilage and sloughed-off border cells (Nguyen, 2009). The exudates have been found to play important role in soil ecology and plant nutrition. For example, they have been found to enhance mycorrhizal fungal growth (Elias and Safir, 1987; Ratnayake et al., 1978; Tawaraya et al., 1996) thereby helping the plant to explore larger volumes of soil in search of nutrients. Moreover, organic acids present in exudates, help solubilize the insoluble phosphorus (P) in rhizosphere (Moghimi et al., 1978; Lipton et al., 1987; Saleque and Kirk, 1995). This knowledge has even led to the development of

novel plant varieties more efficient in using soil P through genetically-engineered enhanced release of organic acids by roots (López-Bucio et al., 2000). Finally exudates have also been suggested to increase the SOM turnover by promoting the microbial activity in the rhizosphere (Hamilton and Frank, 2001; Phillips et al., 2011) although the direct evidence is still lacking (Jones et al., 2004).

The mineralization of SOM is accelerated under living plants when compared to unplanted controls due to stimulation of soil microbes (Helal and Sauerbeck, 1984; Liljeroth et al., 1994; Kuzyakov and Cheng, 2001) - a phenomenon known as the rhizosphere priming effect (RPE). It has been suggested that exudates from roots and root-associated mycorrhizae provide energy-rich substrates to rhizosphere microbes thereby enabling them to secrete extracellular enzymes responsible for the accelerated SOM decomposition (Clarholm, 1985; Hamilton and Frank, 2001). However, lab incubations testing the effect of various components of exudates on SOM mineralization have reported contradictory results. Briefly, the additions of isotopically labeled sugars, amino acids and organic acids induced positive or negative priming effects (Hamer and Marschner, 2005a, 2005b; Blagodatskaya et al., 2007; Ohm et al., 2007) or did not have any effect even if the microbial activity was stimulated (De Nobili et al. 2001; Salomé et al. 2010). This lack of effect is explained by *r*-strategist microorganisms that only use these easily degradable substrates (Fontaine et al., 2003; Blagodatskaya et al., 2007; Ohm et al., 2007). Despite their association with roots of about 80% of terrestrial plant species, the role of arbuscular mycorrhizal fungi (AMF) in the RPE remains unknown. The catabolic capabilities of root-associated AMF are generally considered low compared to those of soil decomposers (Read and Perez-Moreno, 2003; Talbot et al., 2008), suggesting they probably play a minor role in rhizosphere priming. However, they have been found to participate in the degradation of plant

litter (Hodge et al., 2001; Leigh et al., 2009; Cheng et al., 2012), suggesting that their catabolic capability has been underestimated. Finally, the RPE induced by plants may arise from the supply of litter to soil decomposers since this type of organic matter systematically induces the priming effect in incubation studies (Nottingham et al. 2009; Pascault et al. 2013). Knowledge of the contribution of exudates, AMF and root litter depositions to RPE is fundamental to predicting plant effects on soil C cycling under changing climates.

Plant species differ in terms of total labile C inputs through rhizodeposition into the soil thereby inducing varying stimulation of soil microorganisms and RPE (Dijkstra et al., 2006a,b). Moreover, a specific plant species can shape a specific structure of microbial community (Grayston et al., 1997; Germida et al., 1998; Broeckling et al., 2008) by controlling the quality and quantity of rhizodeposition into the rhizosphere (Grayston et al., 1997; Broeckling et al., 2008). Therefore it is important to study the mechanisms of rhizosphere priming under different plant species and linking the variation in RPE with the soil microbial community structure shaped by a certain plant species.

The aim of this study was to determine the role of exudates and AMF in rhizosphere priming, the relative importance of exudates, AMF and roots in determining the RPE and mechanisms by which each of these induce changes in the RPE. The effect of exudates on SOM dynamics was disentangled from that of mycorrhizae and root-litter deposition by using meshes of different pore sizes under monocultures of three grassland species namely *Lolium perenne* (Lp), *Poa trivialis* (Pt) and *Trifolium repens* (Tr). Continuous  $^{13}\text{C}$  labeling of plants was used to distinguish soil-derived (Rs) and plant-derived respiration (Rp). The rhizosphere priming effect was calculated as the difference between Rs from planted soils and from control bare soil. We

hypothesized that exudates and root litter would induce strong priming effects by favoring the growth of microbes and AMF would not have any positive effect on RPE.

## 2. Materials and Methods

### 2.1. Soil sampling and conditioning

The soil was sampled from an upland grassland located in the environmental research observatory (ORE) established by French National Institute for Agricultural Research (INRA) in central France in 2003 (Theix, 45° 43'N, 03°01'E). The soil is a drained Cambisol developed from a granitic rock. The soil was taken from 10-40 cm soil profile. The upper 10 cm that is rich in fresh C was removed given that respiration of this pre-existing unlabeled fresh C cannot be separated from that of recalcitrant SOM. Moreover the presence of plants can modify the decomposition of fresh C (Personeni and Loiseau, 2004). The soil properties were: pH  $6.3 \pm 0.23$ , clay  $21 \pm 2.1$  %, soil organic carbon (SOC)  $17 \pm 0.28$  g kg<sup>-1</sup> soil and SOC  $\delta^{13}\text{C}$   $-26.4 \pm 0.02$  ‰. This soil was enclosed in small PVC cylinders (height 1.5 cm, diameter 5 cm) whose sides were sealed by the mesh of three different pore sizes. The pore size 0.45  $\mu\text{m}$  was meant to only allow the entry of exudates excluding the mycorrhizae and roots in a living rhizosphere (exudates treatment). Whereas, the pore sizes 30 and 1000  $\mu\text{m}$  would also permit the entry of mycorrhizae and roots (mycorrhizae & roots) respectively. From now on, these soil-containing cylinders will be called soil compartments.

### 2.2. Establishment of monocultures

Three grassland species i.e. *Lolium perenne* (Lp), *Poa trivialis* (Pt) and *Trifolium repens* (Tr), that were previously found to induce variable priming effects (Shahzad et al., 2012), were selected for this experiment. PVC pots (20 cm high, 7.8 cm internal diameter) were filled with sand (pH 7) whereas four soil compartments each containing about 15g of equivalent dry soil were placed vertically in each pot (Fig. 1). In August 2010, twelve pots were sown by each of the



three plant species representing four replicates for each pore size of mesh (0.45, 30, 1000  $\mu\text{m}$ ). Four pots containing soil compartments (1000  $\mu\text{m}$  mesh) were kept bare as control soil. An automated drip irrigation method was used for water supply and all pots were water-saturated whenever the soil moisture decreased to  $75 \pm 5 \%$  of the soil field capacity. The near-field capacity conditions were maintained to avoid the artifact of drying-rewetting cycles that may increase  $\text{CO}_2$  release from soil (Schimel et al., 2011).

### *2.3. Labeling system*

The detailed description of the labeling system has previously been given in Shahzad et al., 2012 (Materials & Methods and Supplementary material). Briefly, a compressor injected ambient air into a molecular sieve to remove  $\text{CO}_2$ ,  $\text{H}_2\text{O}$  and all particles. The  $\text{CO}_2$ -free air was then mixed with  $^{13}\text{C}$ - depleted  $\text{CO}_2$  of fossil fuel origin ( $\delta^{13}\text{C}$ :  $-38.55 \pm 0.07 \text{‰}$ ) and passed through a humidifier. The water flow in humidifier was regulated such that the relative humidity of the labeled air reaching the chamber, containing all the microcosms (soil, plants) used in this experiment, was around 50-60%. The air injected in the chamber had a  $\text{CO}_2$  concentration of  $400 \pm 20$  ppm. The chamber consisted of an iron box with a plexiglass screen mounted on it (dimensions:  $350 \times 140 \times 140$  cm). All planted and bare pots were placed in the chamber and continuously ventilated with air produced by labeling system. The volume of the air in the chamber was renewed twice a minute. This quick renewal of the air was used to maintain constant concentration (400 ppm) and isotopic composition ( $-38.55 \text{‰}$ ) of  $\text{CO}_2$  in the chamber. The ventilation of air did also not let the temperature difference exceed  $2 \text{ }^\circ\text{C}$  between inside and outside of chamber. All the planted and bare pots were placed in a continuous  $^{13}\text{C}$ - $\text{CO}_2$  labeling system throughout the experiment.

#### *2.4. Mycorrhizal colonization*

Since the plants were grown in sand which is an unfavorable medium for the spontaneous development of mycorrhizal colonization of roots, an inoculum of endomycorrhizae was applied to plant pots. Viable fungal spores were extracted in 1% CaCl<sub>2</sub> on a filter paper using fifty grams of fresh soil, taken from the same field from where experimental soil was taken. The number of viable fungal spores was found sufficient during trials of extractions i.e. at least 200 per filter paper. The filters containing spores were gently washed with distilled water at the base of the one week-old plants. It should be noted that all the plants irrespective of mesh treatments were inoculated. Murashige and Skoog (1962) nutritive solution (MS0, without sucrose) was applied thrice during the experiment: first application was done without nitrogen salts one day after the inoculation. Briefly 100 ml of salt concentrations namely calcium chloride (400 mg/L), magnesium sulphate (370 mg/L), Potassium phosphate (170 mg/L) and Sodium molybdate (0.25 mg/L) were added to each pot. The following applications were done two and five days after inoculation and included the nitrogen concentrations i.e. Ammonium nitrate (1650 mg/L) and Potassium nitrate (1900 mg/L). The nutritive solutions were applied to all the pots irrespective of mesh treatments.

Mycorrhizal colonization was measured using the method of McGonigle et al., (1990). Briefly, roots of 5 plants at the end of experiment were cut into smaller pieces of about 1 cm each, cleared in 10% KOH during 24 h, rinsed with distilled water, acidified with 1% HCl during 15 minutes, stained overnight with 0.1% Trypan blue at ambient temperature, rinsed with distilled water and destained in 50% glycerol. About 200 fragments of one sample were mounted on microscopic slides and examined with intersection method at  $\times 200$  magnification. The

parameters measured were arbuscular colonization, vesicular abundance, hyphal colonization and the root fragments non-colonized by mycorrhizae.

### 2.5. Respiration measurements

The evening before each respiration measurement, the pots were irrigated to field capacity thus ensuring that all the respiration measurements were done in similar soil moisture conditions. The next day pots were taken out of the chamber 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<sub>2</sub>. The CO<sub>2</sub> released by soil plant system was trapped in soda lime trap of 100 ml of 1M NaOH that was placed in the respiration chambers. By conducting additional measurements in the respiration chambers using chromatography, we found that the soda lime trap fixed more than 99% of the CO<sub>2</sub> released over 24 hours by soil-plant system. Total C trapped in NaOH was measured using total inorganic C analyzer. Carbonates in NaOH of a subsample were precipitated using an excess BaCl<sub>2</sub> and filtered. The <sup>13</sup>C abundance of carbonates (trapped CO<sub>2</sub>) was measured with an elemental analyzer coupled to an Isotope-ratio mass spectrometer (IRMS). The soil-derived CO<sub>2</sub> ( $R_s$ , mg C-CO<sub>2</sub> kg<sup>-1</sup> dry soil day<sup>-1</sup>) was separated from plant-derived CO<sub>2</sub> ( $R_p$ , mg C-CO<sub>2</sub> kg<sup>-1</sup> dry soil day<sup>-1</sup>) using mass balance equations:

$$R_t = R_s + R_p \quad \text{Eq. 2.5.1}$$

$$R_t \times A_{tr}^{13} = R_s \times A_s^{13} + R_p \times A_p^{13} \quad \text{Eq. 2.5.2}$$

Where  $R_t$  was the total CO<sub>2</sub> emitted by the plant-soil system,  $R_s$  was the soil-derived CO<sub>2</sub> released as result of microbial mineralization of SOM,  $R_p$  was the CO<sub>2</sub> coming from plant,

mycorrhizae and microbial respiration of rhizodeposits and plant litter,  $A_s^{13}$  was the  $^{13}\text{C}$  abundance (% atom) of soil-derived carbon,  $A_p^{13}$  was the  $^{13}\text{C}$  abundance of respective plant root C and  $A_{tr}^{13}$  was the  $^{13}\text{C}$  abundance of total  $\text{CO}_2$  emitted from the plant-soil system. Equations 2.5.1 and 2.5.2 were resolved to calculate soil-derived  $\text{CO}_2\text{-C}$  from total  $\text{CO}_2$  emitted from soil-plant system as follows:

$$R_s = R_t \times (A_{tr}^{13} - A_p^{13}) / (A_s^{13} - A_p^{13}) \quad \text{Eq. 2.5.3}$$

The rhizosphere priming effect (RPE,  $\text{mg CO}_2\text{-C kg}^{-1}$  dry soil  $\text{day}^{-1}$ ) induced by the plants was calculated as:

$$RPE = (R_s, \text{planted soil}) - (R_s, \text{control soil}) \quad \text{Eq. 2.5.4}$$

Where  $R_s$ , control soil ( $\text{mg C-CO}_2 \text{ kg}^{-1}$  dry soil  $\text{day}^{-1}$ ) was  $\text{CO}_2$  emitted by bare control soil.

## 2.6. Plant & soil analyses

Fifty one days after sowing, pots were destructively sampled for plant and soil analysis. Soil from the four compartments of each pot was taken out and mixed homogenously. Plant roots were washed to remove the sand and the soil (for  $>1000 \mu\text{m}$  mesh) attached to them. Roots and shoots were then oven dried for 48 h at  $60 \text{ }^\circ\text{C}$  and finely ground. The dried plant material was analyzed on an elemental analyzer coupled to an isotope ratio mass spectrometer (IRMS) for total C and N content and  $^{13}\text{C}$  abundance. Microbial biomass was measured using a modified version (Fontaine et al., 2011) of the fumigation-extraction method proposed by Vance et al., (1987). Briefly 5 g of soil was extracted with 20 mL of 30mM  $\text{K}_2\text{SO}_4$  after 1 hour shaking. Another 5 g of soil sample was fumigated with alcohol free chloroform under vacuum conditions

in a glass desiccator for 24 hours. Chloroform was removed from the soil by ventilation and soils were extracted with 20 mL of 30mM K<sub>2</sub>SO<sub>4</sub>. The extracts were filtered (0.45 μm) and then lyophilized. The recovered crystals were analyzed for C content and δ<sup>13</sup>C. Total microbial biomass ( $MB_t$ ) was calculated as:

$$MB_t = \frac{1}{k}(C_f - C_{nf}) \quad \text{Eq. 2.6.1}$$

Where  $C_f$  and  $C_{nf}$  were the carbon content of crystals obtained from extraction of fumigated and non-fumigated soil samples, respectively, and  $k$  is extraction yield of microbial biomass ( $k = 16\%$ , Fontaine et al., 2004b). The soil-derived ( $MB_s$ ) and plant-derived ( $MB_p$ ) microbial biomasses were determined as:

$$MB_t = MB_p + MB_s \quad \text{Eq. 2.6.2}$$

$$MB_t \times A_{t\text{bm}}^{13} = MB_p \times A_p^{13} + MB_s \times A_s^{13} \quad \text{Eq. 2.6.3}$$

Where  $A_{t\text{bm}}^{13}$  was the <sup>13</sup>C abundance of total microbial biomass. The  $A_{t\text{bm}}^{13}$  was calculated as:

$$A_{t\text{bm}}^{13} = (C_f \times A_f^{13} - C_{nf} \times A_{nf}^{13}) / (C_f - C_{nf}) \quad \text{Eq. 2.6.4}$$

Where  $A_f^{13}$  and  $A_{nf}^{13}$  were the <sup>13</sup>C abundances of  $C_f$  and  $C_{nf}$  respectively.

In order to determine if root exudates really penetrated in soil compartments, the amount of plant-derived C incorporated in soil was quantified for the three pore sizes (0.45, 30, 1000 μm) at the end of the experiment. The soil organic C content and its δ<sup>13</sup>C were determined with an elemental analyzer coupled to an IRMS. By adopting the same approach used for CO<sub>2</sub> and

microbial biomass, the amount of plant-derived C incorporated in soil C ( $C_p$ ) was calculated as follows:

$$C_p = C_t \times (A_t^{13} - A_s^{13}) / (A_p^{13} - A_s^{13}) \quad \text{Eq. 2.6.5}$$

Where  $C_t$  was the total soil carbon content and  $A_t^{13}$  its  $^{13}\text{C}$  abundance.

## 2.7. PLFA measurements

A soil sample of 2 g was freeze-dried and ground for each replicate after remaining plant materials were picked out. Phospholipids fatty acids (PLFA) were extracted using a modified method of Bligh and Dyer (1959) (Frostegård et al., 1991). Briefly, PLFA were extracted in a single-phase mixture of chloroform methanol: citrate buffer (1:2:0.8, v:v:v, pH 4.0) shaken at 400rpm for 1h. Phase splitting was done by adding equal volume of chloroform and citrate buffer. The organic phase was then submitted to a solid phase extraction on silica gel extraction cartridges (Discovery® DSC-Si SPE Tube bed wt. 500mg, volume 3mL from Supelco). Neutral lipids, glycolipids and PLFA were eluted by chloroform, acetone and methanol respectively. Methyl nonadecanoate (fatty acid methyl ester 19:0) was added as an internal standard and PLFA were trans-methylated under mild alkaline conditions to yield fatty acid methyl esters (FAMES), (Dowling et al., 1986). FAMES were then analyzed by GC/MS (4000 GC/MS, Varian) in split-less mode (1 mL, injector temperature: 250 °C) equipped with a BPX70 column (60m, 0.25 mm i.d., 0.25 mm df., SGE), and helium as a carrier gas. The temperature program was 50 °C for 5 min, raised to 165 °C at 15 C/min, followed by increases of 2 C/min up to 225 °C. This temperature was held for 15 min. To identify the FAMES, the retention times and mass spectra were compared with those obtained from standards (Bacterial Acid Methyl Ester Mix from Supelco and 11 Hexadecenoic acid (92 % cis. 8% trans) from Matreva). The PLFAs i15:0, a15:0,

i16:0, and i17:0 were designated as derived from gram positive bacteria while 17:0cy, 19:0cy & 16:1 $\omega$ 9c were designated as derived from gram negative bacteria (Frostegård and Bååth, 1996; Zelles, 1997). The PLFAs 18:1 $\omega$ 9c, 18:2 $\omega$ 9t and 18:2 $\omega$ 6c were designated as representatives of saprophytic fungi (Frostegård and Bååth, 1996; Zelles, 1997). The PLFA 16:1 $\omega$ 5c are often considered to represent the arbuscular mycorrhizal fungi (Olsson et al., 1995) although they can also be found in bacteria (Nichols et al., 1986). For this study this biomarker is being considered a representative of arbuscular mycorrhizal fungi (AMF) albeit with caution as suggested recently by Frostegård et al., (2011).

## 2.8. Statistical analyses

A two way analysis of variance (ANOVA) (95% CI) was used to determine the significant effect of plant species and mesh treatment on soil organic matter mineralization i.e. soil-derived CO<sub>2</sub>-C and rhizosphere priming effect. The relationship between plant-derived CO<sub>2</sub>-C or plant biomass and the rhizosphere priming effect across the plant species and mesh treatments was assessed by simple regression analysis. The effect of mesh treatment on total soil microbial biomass, soil-derived microbial biomass, plant-derived microbial biomass and different microbial groups was determined using one-way ANOVA. One-way ANOVA was also used to determine the significant effect of mesh treatment on RPE efficiency i.e. RPE induced per unit of plant-derived C assimilated in microbial biomass. All statistical analyses were performed with *Statgraphics Plus* (Manugistics, USA).

### 3. Results

All the three grassland species induced strong rhizosphere priming effects across all the mesh treatments ( $P < 0.05$ , Fig. 2). The SOM mineralization ( $R_s$ ) in planted soils remained consistently higher throughout the incubation, representing between 118 % and 640 % of respiration observed in bare soil. The three plant species significantly varied in terms of the RPE induced ( $P < 0.05$ , Fig. 2), with the highest RPE induced by *Trifolium repens* (Tr), followed by *Lolium perenne* (Lp) and *Poa trivialis* (Pt) respectively. A strong correlation was found between the RPE and plant-derived CO<sub>2</sub>-C ( $R_p$ ,  $r^2 = 0.79$ ) or total plant biomass across all the species ( $r^2 = 0.59$ , Fig. 3).

Initially the mesh pore size (0.45, 30 or 1000  $\mu\text{m}$ ) had no effect on RPE (Fig. 2) indicating that all the RPE across the three plant species was induced by the root exudates. The roots might not have yet grown enough to reach in soil compartments. However during later stages of the experiment, the presence of roots (1000  $\mu\text{m}$  mesh) in soil compartments significantly increased the total RPE when compared to that due to exudates alone (0.45  $\mu\text{m}$  mesh) ( $P < 0.05$ , Fig. 2). The contribution of roots in induced RPE (% of the total) was 35-42 % for Lp, 20 to 28 % for Pt and 26% for Tr. The 30  $\mu\text{m}$  mesh treatment, in which AMF could pass through linking roots and soil compartments, showed no effect on SOM mineralization in addition to that by exudates under all the plant species throughout the experiment ( $P > 0.05$  Fig. 2).

Significant amounts of plant-derived C (<sup>13</sup>C labeled C) were added to total soil organic carbon in mesh treatments 0.45  $\mu\text{m}$  and 30  $\mu\text{m}$  under all the three plant species. The amount of plant C incorporated in soil organic carbon in mesh treatments 0.45  $\mu\text{m}$  and 30  $\mu\text{m}$  was 153±19



and  $150 \pm 8$ ,  $147 \pm 14$  and  $159 \pm 27$  and  $169 \pm 27$  and  $163 \pm 22$  mg C kg<sup>-1</sup> soil for Lp, Pt and Tr respectively. However the presence of roots induced incorporation of highest amounts of plant-derived C in soil organic C in the presence of roots (Fig. S2) that were  $1892 \pm 256$ ,  $681 \pm 120$  and  $1463 \pm 229$  mg C kg<sup>-1</sup> soil for Lp, Pt and Tr respectively.

The three plant species showed varying degree of arbuscular mycorrhizal root colonization (%) which also varied with mesh pore size (Table S1, Supplementary Material). No colonization was found for Lp for all mesh pore sizes and Pt for 0.45  $\mu$ m mesh. The Pt showed a root colonization of 4.56 % (Coefficient of variation, CV = 0.32 %) and 6.68 % (CV = 0.46 %) for 30  $\mu$ m and 1000  $\mu$ m mesh pore size respectively. The overall mycorrhizal root colonization was higher in Tr than the other plant species with percentage colonization of 8.55 % (CV = 0.85 %), 16.98 % (CV = 0.46 %) and 17.54 % (CV = 0.67) for 0.45  $\mu$ m, 30  $\mu$ m and 1000  $\mu$ m mesh pore sizes respectively.

Total microbial biomass ( $MB_{tot}$ ) remained unchanged under 0.45  $\mu$ m and 30  $\mu$ m mesh treatments under all plant species ( $P > 0.05$ ) except Pt where  $MB_{tot}$  increased by 27.9 ( $\pm 24.5$ ) % in the presence of exudates i.e. 0.45  $\mu$ m mesh ( $P < 0.05$ , Fig. 4). In contrast, the presence of roots in soil compartments (1000  $\mu$ m mesh) significantly increased the  $MB_{tot}$  under all species ( $P < 0.05$ ). The  $MB_{tot}$  was almost doubled in the presence of roots of Lp and Tr. The soil C-derived microbial biomass ( $MB_{soc}$ ), remained unchanged under 0.45  $\mu$ m and 30  $\mu$ m mesh treatments under all plant species when compared to control soils ( $P > 0.05$ ) except Pt where it significantly increased in the presence of exudates ( $P < 0.05$ ). However, the presence of roots in soil compartments significantly increased  $MB_{soc}$  in comparison to control soils under all the plant species ( $P < 0.05$ ) except Pt where it remained unchanged ( $P > 0.05$ ). The assimilation of plant carbon (<sup>13</sup>C labeled C) i.e. plant-derived microbial biomass ( $MB_{plant}$ ) was significant in all mesh

treatments under all the plant species ( $P < 0.05$ , Fig. 4). In mesh treatments 0.45  $\mu\text{m}$  and 30  $\mu\text{m}$ ,  $23 \pm 1.6$  and  $45.1 \pm 9.2$ ,  $21.9 \pm 2.6$  and  $32.1 \pm 7.6$  and  $25.7 \pm 3.4$  and  $28.2 \pm 8.5$  mg plant-derived C  $\text{kg}^{-1}$  soil was assimilated by microbial biomass under Lp, Pt and Tr respectively. The plant-derived microbial biomass was significantly higher in 30  $\mu\text{m}$  than 0.45  $\mu\text{m}$  mesh treatment in Lp and Pt. However the presence of roots (1000  $\mu\text{m}$  mesh) increased  $\text{MB}_{\text{plant}}$  by 2 (Pt) to 9 times (Tr) when compared to 0.45  $\mu\text{m}$  and 30  $\mu\text{m}$  mesh treatments ( $P < 0.05$ ).

The plant C derived microbial biomass ( $\text{MB}_{\text{plant}}$ ) was used to determine RPE efficiency i.e. the amount of RPE induced per unit of plant-derived C assimilated into microbial biomass. The RPE efficiency was highest in the presence of exudates or root-associated AMF while it was lowest in the presence of roots for all the species (Fig. 5). For example, root exudates were 3 (Pt), 6 (Lp) or 7 (Tr) times more efficient than the roots in inducing the RPE. Under Lp, significantly lower RPE efficiency was found in 30  $\mu\text{m}$  mesh treatment than that in the presence of exudates (0.45  $\mu\text{m}$  mesh) but it was still higher than that in the presence of roots.

The AMF biomarker (PLFA 16:1 $\omega$ 5c) in the soils showed no change under 0.45  $\mu\text{m}$  (exudates) and 30  $\mu\text{m}$  (permeable to root colonizing mycorrhizae) mesh treatments under all the plant species ( $P > 0.05$ ). The presence of roots (1000  $\mu\text{m}$  mesh) significantly increased the concentration of AMF in soil but only under Tr (Fig. 6). The concentrations of all saprophytic microbial groups remained unchanged in 0.45  $\mu\text{m}$  and 30  $\mu\text{m}$  mesh treatments under all the plant species ( $P > 0.05$ ). However, the presence of roots increased their concentrations by large amounts across all the species ( $P < 0.05$ ). The response of Gram negative bacteria to various types of plant C deposition was inconsistent under the three plant species (Fig. 6). The concentrations of Gram negative bacteria, when compared to that in control soils, were significantly increased in the presence of exudates under Lp and Tr but it remained unchanged

under Pt. However, their concentrations remained unchanged for 30  $\mu\text{m}$  mesh. Moreover, the presence of roots stimulated concentrations of Gram positive bacteria only under Pt and Tr but not Lp.

#### 4. Discussion

The three perennial plant species induced substantial over-production of unlabeled CO<sub>2</sub>. The extent of this rhizosphere priming is similar to other studies (Cheng, 2009; Dijkstra and Cheng, 2007; Zhu and Cheng, 2011). The RPE can have two origins: i/ an increase in SOM mineralization and ii/ an acceleration of microbial turnover (Bingeman et al., 1953; Dalenberg and Jager, 1989, 1981). Indeed, the supply of labeled C may activate dormant microbes which renew their metabolites and release unlabeled microbial C as CO<sub>2</sub>. The over-production of unlabeled CO<sub>2</sub> in this case is often called apparent RPE because it comes from an acceleration of microbial turnover and not from mineralization of SOM (Bingeman et al., 1953; Dalenberg and Jager, 1989, 1981). The occurrence of apparent RPE can be detected by measuring the amount of unlabeled C in microbial biomass of control and C-amended soil; the apparent PE decreases the unlabeled microbial C in the C-amended soil (Wu et al., 1993; Fontaine et al., 2004b). Our results show that the presence of plants had no effect or increased the unlabeled microbial C (Fig 4), indicating that the observed RPE was mostly the result of an increase in SOM mineralization i.e. the apparent RPE is negligible compared to the real RPE.

Most of the root biomass for all the plants was concentrated around soil compartments covered by meshes (Fig. 1) suggesting that the roots were injecting carbon inside the soil compartments and benefiting from the nutrient release by soil microorganisms even in treatments where if the roots were excluded from soil (treatments 0.45  $\mu$ m and 30  $\mu$ m). Indeed, the root exudates are known to diffuse up to 12 mm from their point of release (Sauer et al., 2006) whereas the maximum distance exudates had to cover in this experiment from their entry through mesh of either side to the middle of the soil compartment was 7.5 mm (height of the PVC cylinder used was 1.5 cm) indicating that the whole soil present in a compartment was accessible

to exudates. This diffusion of exudates into soil compartments is confirmed by the significant amounts of plant-derived C present in soil organic carbon (Fig. S2) and microbial biomass (Fig. 4). Therefore the SOM mineralization and other soil attributes in 0.45  $\mu\text{m}$  mesh treatment can safely be attributed to exudates.

Our findings suggest that root exudates are the most important means for a plant to induce an acceleration in SOM mineralization i.e. rhizosphere priming effect (Fig. 2). The result was common for one leguminous and two grasses although they have different growth strategies (Maire et al., 2009). The role of exudates in the stimulation of microbial enzyme activities and SOM mineralization has previously been suspected (Hamilton and Frank, 2001; Phillips et al., 2012). However, by disentangling the effect of exudates from other root-induced processes, the fact that the SOM mineralization is mainly driven by exudates has important consequences for our understanding of plant-soil interactions. The deposition of exudates is closely connected to the plant photosynthetic activity, with a transfer of photosynthates to rhizosphere microorganisms occurring in less than 24 hours (Johnson et al., 2002; Deneff et al., 2009; De Deyn et al., 2011). Given that these exudates drive the SOM mineralization and thereby the release of mineral N (Phillips et al., 2011; Dijkstra et al., 2013), the plant might finely adjust its own N supply to the potential growth offered by its environment (e.g. light,  $\text{CO}_2$ ). The fine control of SOM mineralization by plants is supported by a recent study (Shahzad et al., 2012) showing that the decrease in plant photosynthesis induced by clipping resulted in a 20-56% decrease in RPE within 24 hours.

The plant biomass and plant-derived C respiration, two proxies of plant photosynthesis and exudation (Bahn et al., 2009), were positively linked with RPE across the three plant species (Fig. 2). Therefore, the interspecific differences in RPEs can be explained by the difference of

photosynthetic activity and exudation among the three plant species. The more a genotype is adapted to the environmental conditions, the more photosynthesis it carries out resulting in more C being exuded from roots thereby inducing higher RPE. These results suggest that the fertility of soils (defined here as SOM mineralization) not only depends on inherent properties of soils (i.e. SOM content) but also on plant-soil interactions allowing a fine tuning of SOM mineralization to plant demand which can vary with plant species.

Only *Trifolium repens* (Tr) among the three plant species showed mycorrhizal root-colonization ( $8.55\pm 0.85\%$ ,  $16.9\pm 0.5\%$  and  $17.5\pm 0.7\%$  for mesh  $0.45\ \mu\text{m}$ ,  $30\ \mu\text{m}$  and  $1000\ \mu\text{m}$  respectively, Table S1, Supplementary Material) comparable to previously reported value for this species (20 %, Medina et al., 2010). *Poa trivialis* (Pt) showed significant mycorrhizal root colonization in  $30\ \mu\text{m}$  ( $4.56\pm 0.32\%$ ) and  $1000\ \mu\text{m}$  mesh ( $6.68\pm 0.46\%$ ) treatments whereas no root-colonization was observed in  $0.45\ \mu\text{m}$  treatment. To our knowledge, there is no study that previously reported the mycorrhizal colonization in Pt. Both Pt and Tr showed significantly higher root colonization in  $30\ \mu\text{m}$  and  $1000\ \mu\text{m}$  mesh treatments compared to  $0.45\ \mu\text{m}$  mesh indicating that the root colonization was higher when the mycorrhizal hyphae could access the soil through  $30\ \mu\text{m}$  or  $1000\ \mu\text{m}$  mesh. The better mycorrhizal root colonization of Tr can be explained by the fact that legumes are generally better colonized by mycorrhizal fungi than grasses due to their higher need in P and thereby dependence on AMF for P acquisition (Chen et al., 2005; Eschen et al., 2013). No apparent reason was found for the complete failure of mycorrhizal colonization under Lp.

Under Tr, where root colonization was quantitatively very important, root associated AMF ( $30\ \mu\text{m}$  mesh) did not affect RPE (Fig. 2). Similarly significant mycorrhizal colonization of Pt in the  $30\ \mu\text{m}$  mesh treatment did not induce any increase in the RPE compared to the  $0.45$

$\mu\text{m}$  treatment, suggesting that AMF has no or negligible effect on SOM mineralization rate. It must be noted that the previous studies reporting the affirmation of catabolic capacities of AMF, albeit decomposition of litter only, did not disentangle the effect of exudates from AMF (Hodge et al., 2001; Cheng et al., 2012). It may be speculated that the AMF-induced decomposition may actually come from effect of exudates delivered through them since their ability to produce extra-cellular enzymes is very limited in comparison to ericoid and ecto-mycorrhizal fungi (Read and Perez-Moreno, 2003). However we suggest that this result must be verified with other plants although it supports the idea that AMF have limited decomposing capacity.

Root exudates and the presence of live and dead roots (1000  $\mu\text{m}$  mesh) strongly differ with respect to their effect on SOM mineralization and microbial biomass (Figs. 2 & 4). The amount of RPE induced per unit of plant-derived C assimilated into microbial biomass is 3 to 7 times higher for exudates than for root litter (Fig. 5). This result indicates that the exuded C is used by microbes mostly to synthesize and release extra-cellular enzymes mineralizing SOM instead of promoting microbial growth (Fig 2) and N immobilization (Fig. 1, Supplementary material). This result supports the idea that the RPE is a sort of indirect co-evolved mutualism between plants and rhizosphere microbes (Cheng et al, 2013). The microbial preference for mineralizing activity over growth when relatively small amounts of resource (exudates) are available may have been in anticipation of bigger amounts of a resource (De Nobile et al, 2001). The bigger amounts become available when roots are also present (1000  $\mu\text{m}$  mesh) allowing them to prefer growth over activity which is evident in little increase in the RPE in 1000  $\mu\text{m}$  mesh treatment (Fig. 2). In addition, the microbes are less growth efficient when only exudates are available i.e. they uptake less plant carbon (Fig. 4). The exudates, though a labile and enriched source of energy like a ‘fast-food’, may soon render microbes nutrient-deficient thereby

driving them to accelerate SOM decomposition (RPE). Whereas root litter a relatively complex substrate provides complete diet more likely to support microbial growth. This indirect mutualistic relationship also enables plants to not only provide energy (i.e. exudates) to soil microorganisms but also control the way of energy is used in order to maximize SOM mineralization and their return on investment (greater nutrient availability).

The absence of a substantial shift in microbial community structure in the presence of exudates and the significant increase in saprophytic fungi in the presence of root litter (Fig 6, Table 2 Supplementary material) confirms differences between the two sources of C deposition *vis-à-vis* their use by the soil microbial community. It seems that exudates are good enough for only stimulating the *k*-strategist microbes (Fontaine et al., 2003) to produce and release extracellular enzymes decomposing SOM. Moreover, the growth of *r*-strategist microorganisms (Fontaine et al., 2003), which do not decompose SOM but can grow rapidly on substrates similar to exudates, is blocked by a factor that could not be identified in this study. In contrast, root litter raised saprophytic fungi specifically the biomarker 18:2w6c across all plants. These saprophytic fungi are considered the actors of the PE induced by plant litter (Fontaine et al., 2011; Shahzad, 2012; Shahzad et al., 2012).

In conclusion, our findings show that the plant strongly modulates SOM mineralization (rhizosphere priming effect) through their exudates. This modulation is likely the result of 100,000s of years of plant-microbe co-evolution which has been realized in an atmosphere where CO<sub>2</sub> concentration mostly fluctuated between 200 and 280 ppm (Barnola et al., 1987). However, the human activities have led to a rapid increase in atmospheric CO<sub>2</sub> concentration currently reaching 400 ppm (Mauna Loa Observatory, 2014), with the possibility of disturbing the exudation rate of plants (Phillips et al., 2011). The mineralization of SOM in ecosystems



exposed to elevated CO<sub>2</sub> is intensified (Langley et al., 2009; Phillips et al., 2012) leading, in several cases, to net decrease in SOM stock (Carney et al., 2007; Langley et al., 2009) and an increase in N leaching (Liu et al., 2008; Hungate et al., 2014). These findings suggest that an increase in CO<sub>2</sub> concentration, by increasing the exudation rate of plants and thereby the RPE leads to a rupture of the synchronization (Perveen et al., 2014) between microbial mineralization of SOM and plant uptake of nutrients. Further experiments are necessary to precise the role of exudates in the plant-microbe synchronization in a changing environment.

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## Figure Legends

Figure 1. Top: The schematic diagram of sand filled pot that was used to grow plants on, containing soil-filled small PVC boxes. Below: The destructive sampling of plant roots and soil from real pot.

Figure 2.  $R_s$  i.e. soil-derived  $\text{CO}_2\text{-C}$  ( $\text{mg C kg}^{-1} \text{ soil day}^{-1}$ ) from planted and bare soils under three grassland species. Error bars represent standard error of means.

Figure 3. Relationship between rhizosphere priming effect (RPE) and (a)  $R_p$ , the  $\text{CO}_2\text{-C}$  coming from plant, mycorrhizae and microbial respiration of rhizodeposits and plant litter ( $\text{mg CO}_2\text{-C kg}^{-1} \text{ soil day}^{-1}$ ) (b) total biomass produced by the plants across all species during final measurement of soil  $\text{CO}_2$  efflux. Pt, *Poa trivialis*; Lp, *Lolium perenne*; Tr, *Trifolium perenne*.

Figure 4. Microbial biomass (MB) in control and planted treatments with three meshes. Blank bar represents total MB in control soils where MB<sub>tot</sub> shows total biomass, MB<sub>soc</sub> soil-derived microbial biomass and MB<sub>plant</sub> plant-derived microbial biomass in planted treatments.

Figure 5. Rhizosphere priming effect efficiency i.e. rhizosphere priming effect induced per unit of plant derived carbon assimilated in microbial biomass, in different mesh treatments under three grassland species.

Figure 6. Concentrations of phospholipids fatty acids of AMF, saprophytic fungi, Gram negative and Gram positive bacteria in planted and control soils as affected by C deposition through root exudates only (0.45 $\mu$  mesh), root exudates and root-associated mycorrhizae (30 $\mu$  mesh) and root exudates plus root-associated mycorrhizae and roots (1000 $\mu$  mesh).

Figure S1. Figure S1. (a) Microbial N ( $\text{mg N kg}^{-1}$  soil) and (b) microbial C/N under three grassland species and mesh treatments as compared to bare soil.

Figure S2: Plant-derived i.e.  $^{13}\text{C}$ -labeled C humified in soil organic matter ( $\text{g C/kg soil}$ ) under the three plant species and different mesh treatments.

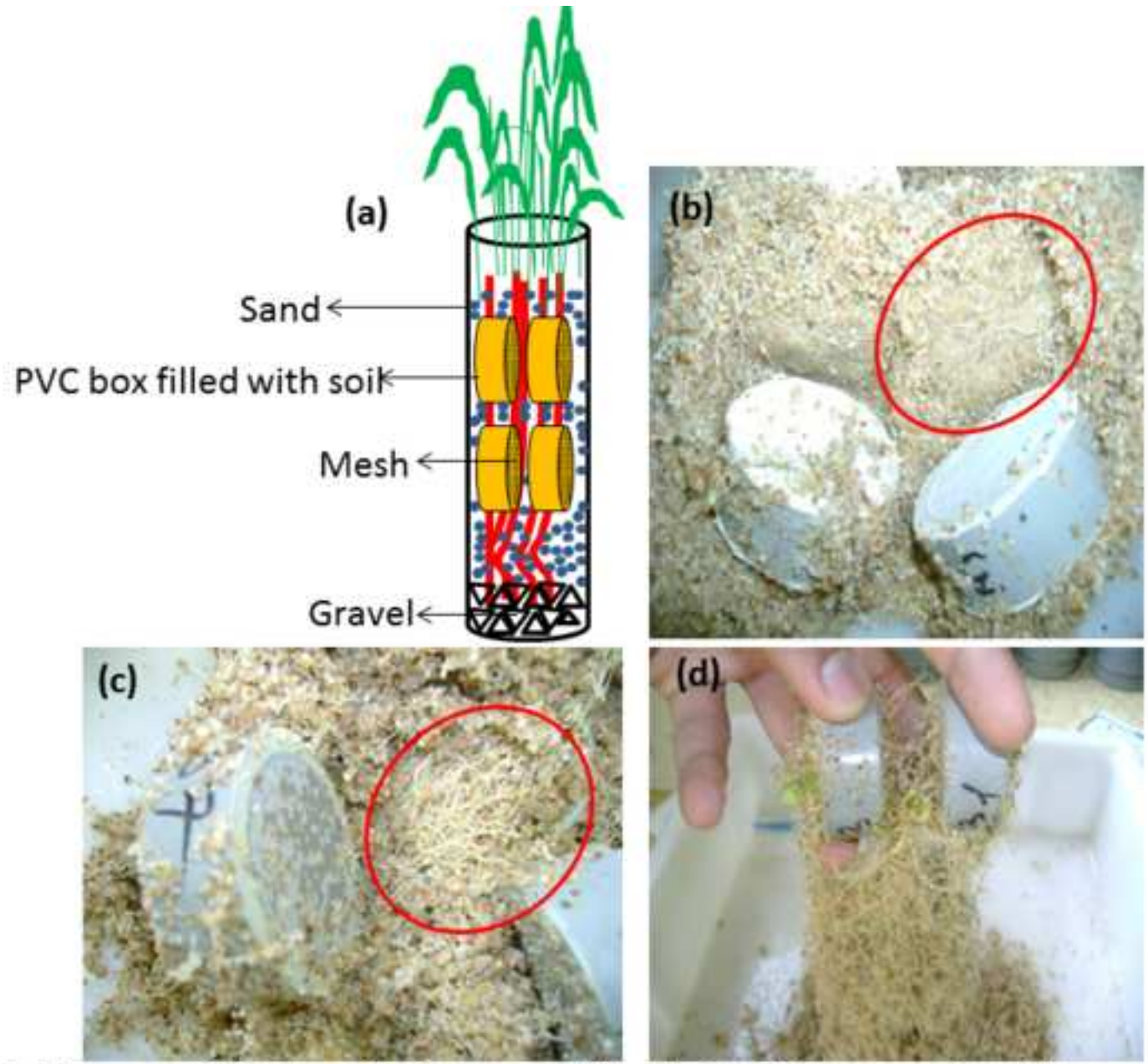


Figure 1. Diagram of plant-soil microcosm (a) . And soil boxes enclosed by mesh of 0.45  $\mu\text{m}$  (b), 30  $\mu\text{m}$  (c) and 1000  $\mu\text{m}$  (d) being taken out from pots. Circles point to assemblage of roots against mesh.

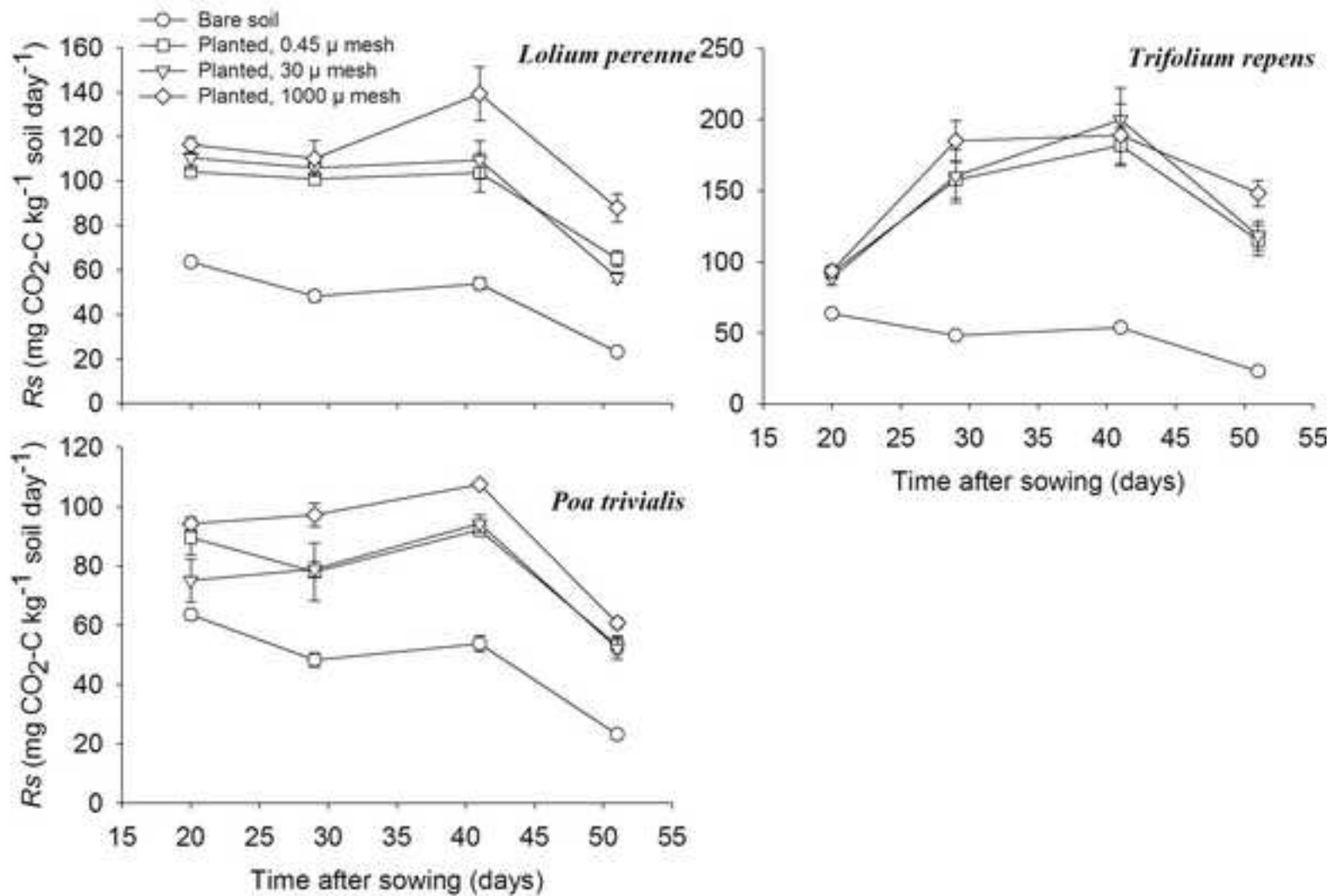


Figure 2.

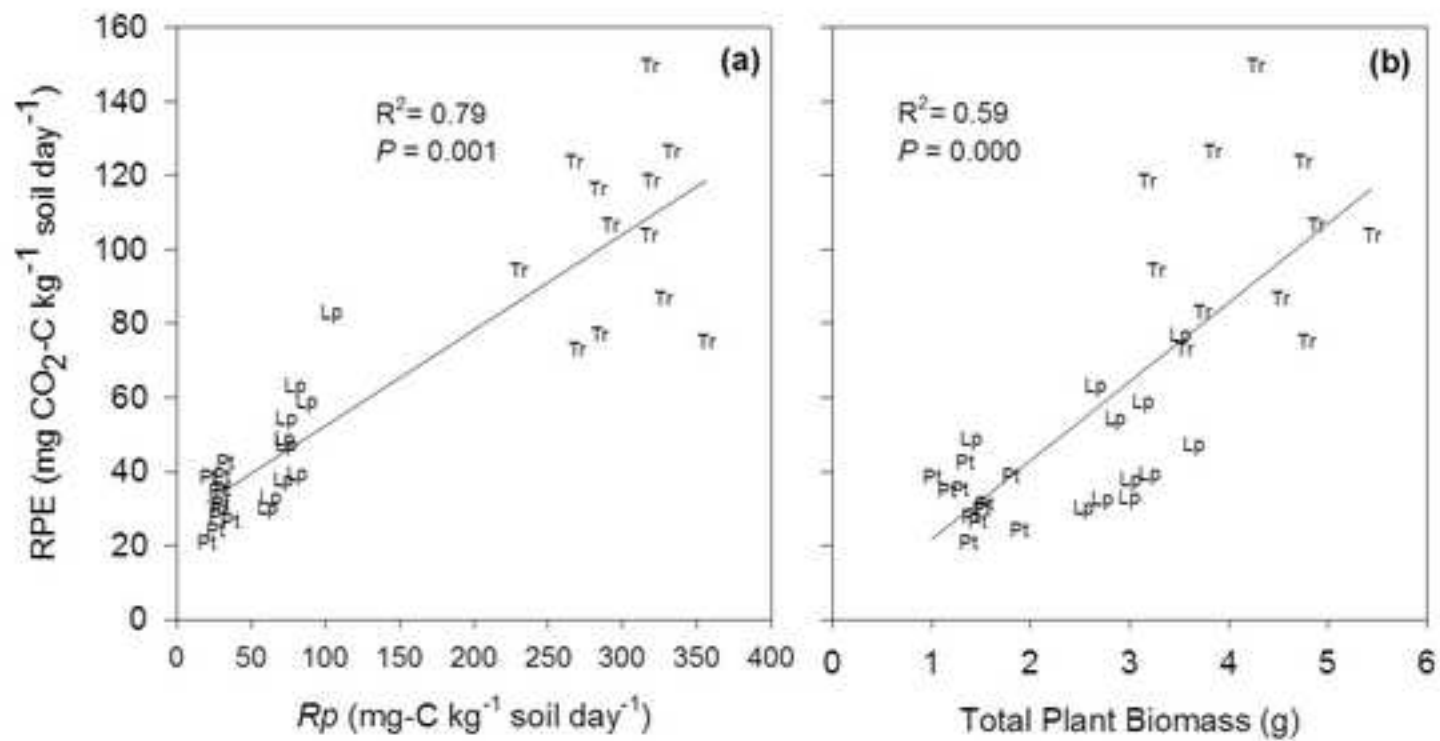


Figure 3.

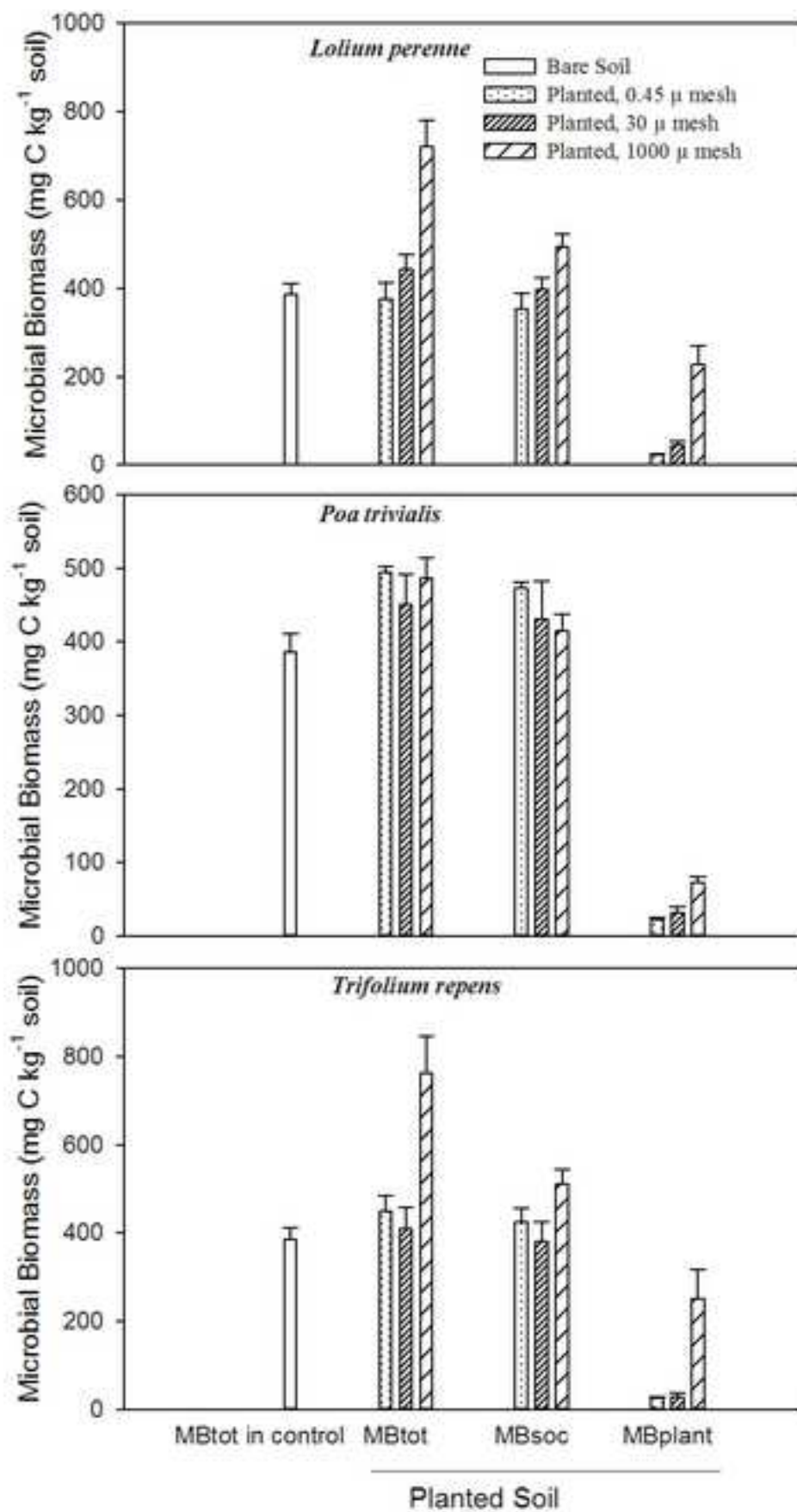


Figure 4.

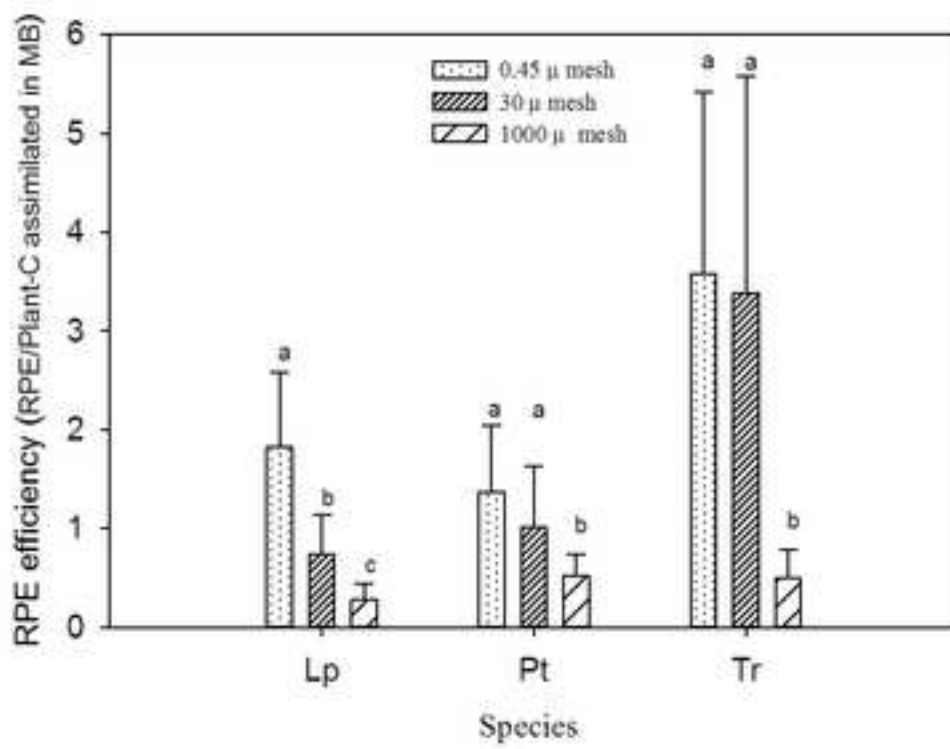


Figure 5.



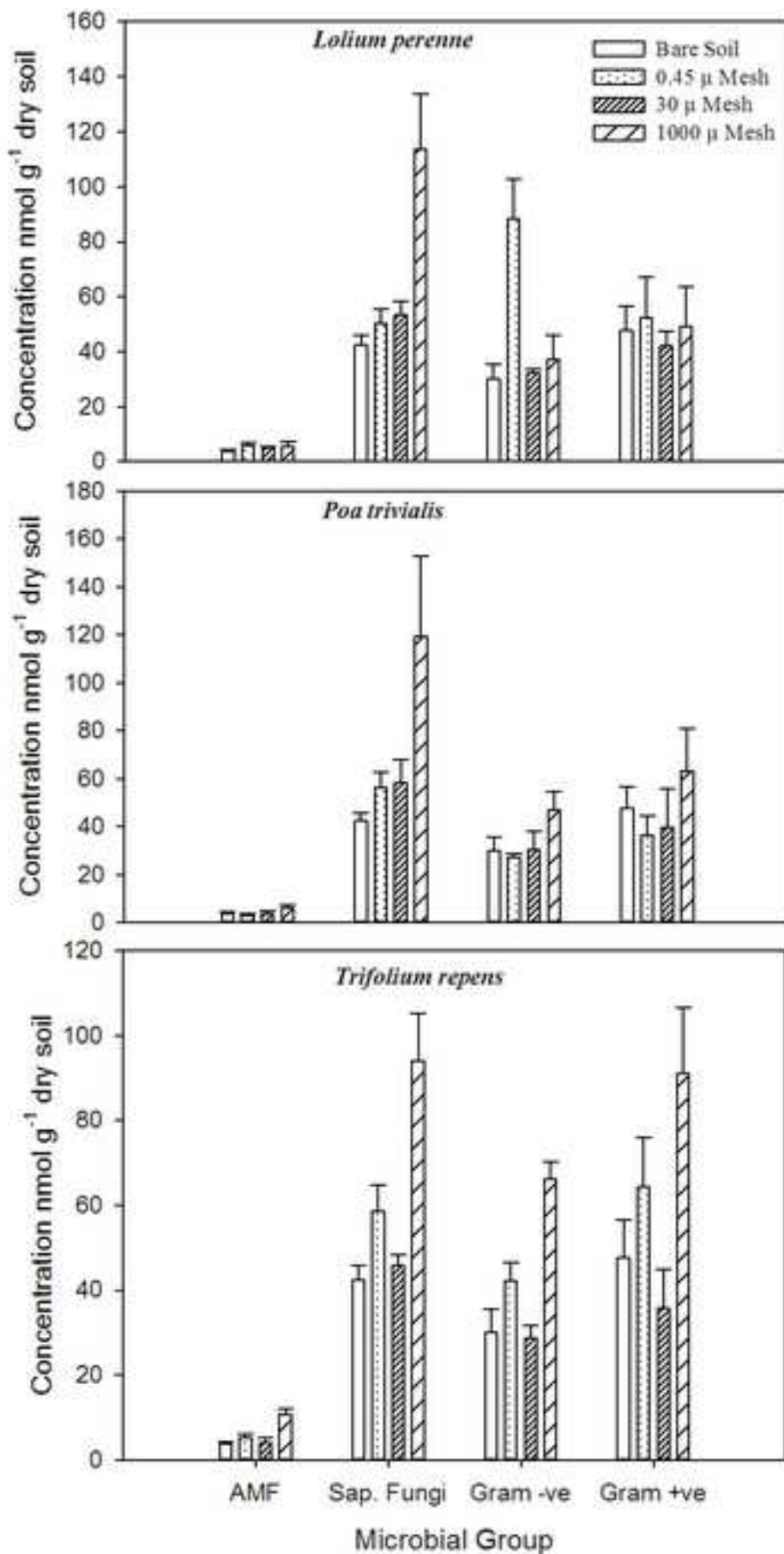


Figure 6.

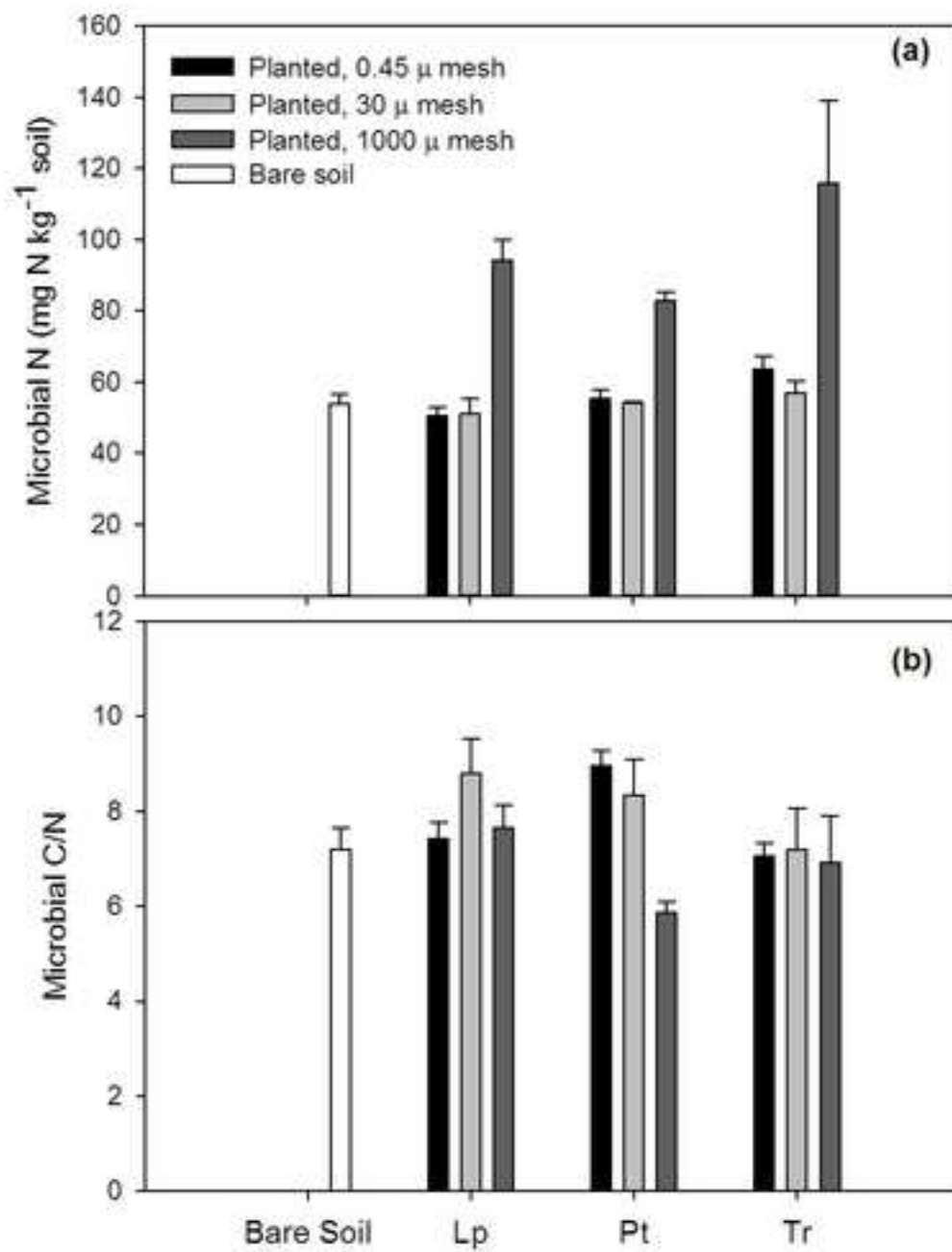


Figure S1.

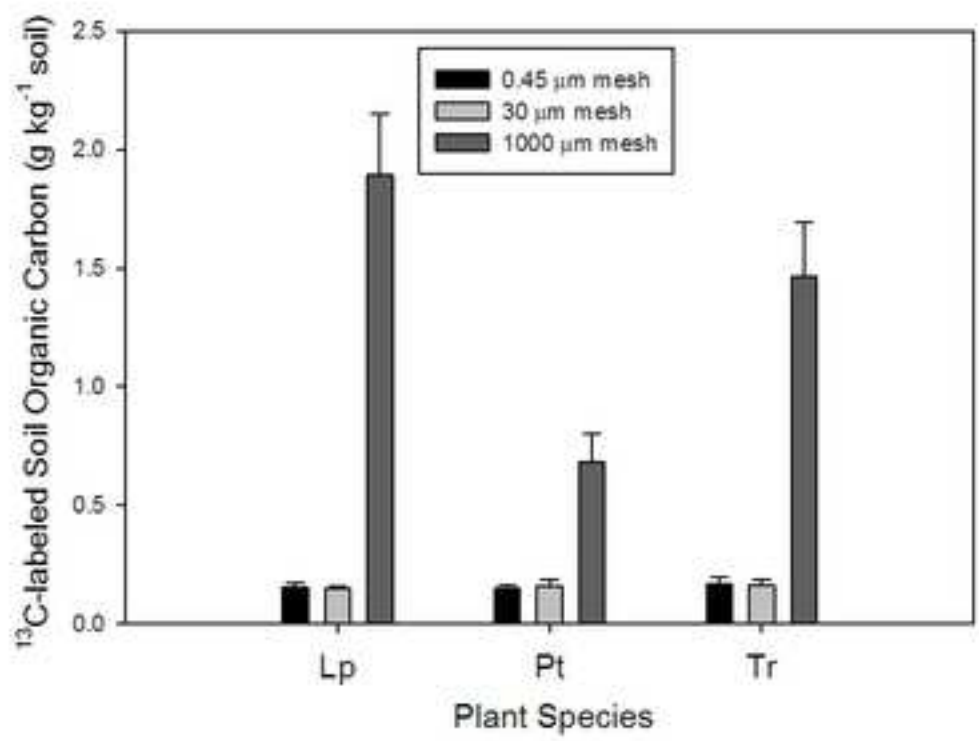


Figure 2S: Plant-derived i.e.  $^{13}\text{C}$ -labeled C humified in soil organic matter ( $\text{g C/kg soil}$ ) under the three species and different mesh treatments.