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Rhizosphere priming effects of *Lolium perenne* and *Trifolium repens* depend on phosphorus fertilization and biological nitrogen fixation

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ABSTRACT

Live roots can stimulate microbial soil organic matter (SOM) decomposition and nutrient cycling, which is termed as the rhizosphere priming effect (RPE). Compared to nitrogen (N) availability, fewer studies have focused on the effect of phosphorus (P) availability on the RPE. Here we investigated the RPEs of ryegrass (*Lolium perenne*) and clover (*Trifolium repens*) with and without P fertilization (4 g P m^{-2}) at three sampling times (Day 30, Day 44, and Day 58 after planting). A continuous ^{13}C–CO_2 labeling method was used to separate soil-derived CO_2 from root-derived CO_2. A nutrient budget method was applied to evaluate the rhizosphere effect on net soil N and P release for plant uptake. We found that ryegrass and clover induced positive RPEs in most plant-soil combinations, ranging from −1% to 134%. Ryegrass exhibited a larger RPE than clover by Day 30, but clover exhibited a larger RPE than ryegrass by Day 44 and Day 58, possibly due to larger shoot biomass regrowth rates, root activity, and rhizodeposition during the later stages. P fertilization significantly decreased the RPE of ryegrass by Day 44 and Day 58, but did not change the RPE of ryegrass by Day 30 and clover at all three sampling times. The reduced RPE of ryegrass with P fertilization was associated with increased microbial biomass N, more root-derived microbial C, and less shoot biomass and root-derived CO_2. These findings suggest that P fertilization coupled with C supply from root exudates induced more microbial N immobilization, which reduced the RPE of ryegrass during later stages when soil N limitation negatively impacted plant growth. However, P-induced microbial N immobilization did not affect clover as much because its biological N fixation, on average 37% of total plant N, may have alleviated soil N limitation. We further observed significant positive relationships between excess net soil N and P release and the RPE by Day 58 across all planted treatments, indicating that soil N and P release by plants can be directly linked to rhizosphere C mineralization. Overall, our results demonstrate the importance of C–N–P interactions for understanding the RPE, which have significant implications for P cycling in plant-soil systems.

1. Introduction

Globally, soil is the largest carbon (C) pool in terrestrial ecosystems, containing more than 1500 Pg organic C in the top 1 m layer (Eswaran et al., 1993; Stockmann et al., 2013) and nearly all nutrients required by plants (McGill and Cole, 1981). Due to the large storage, even a small increase in soil organic matter (SOM) decomposition would cause a positive effect on the global atmospheric CO_2 concentration, thereby exacerbating the global warming induced by fossil fuel burning and land use change (Bond-Lamberty and Thomson, 2010). Besides temperature and water, rhizosphere processes are increasingly recognized as important factors in mediating SOM decomposition and nutrient cycling (Phillips et al., 2011; Finzi et al., 2015).

Plant roots and associated rhizosphere microbes may stimulate or suppress native SOM decomposition, which is termed as the rhizosphere priming effect (RPE) and is an important component of rhizosphere processes (Kuzyakov, 2002; Cheng and Kuzyakov, 2005). Previous studies conducted in growth chambers or greenhouses have shown that

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the magnitude of the RPE could range from a 50% reduction to a 380% enhancement of SOM decomposition as compared to root-free soil (Cheng et al., 2014). These levels suggest that the RPE is a major driver in SOM turnover and nutrient cycling. Therefore, it is essential to understand the potential influencing factors and underlying mechanisms of the RPE for predicting the global C cycle in response to climate change.

Previous studies have shown that the direction and magnitude of the RPE can be significantly influenced by plant species and soil variables (Cheng et al., 2014; Huo et al., 2017). Among soil variables, soil N availability (and N fertilization) has been shown to be closely related to the RPE (Hoosbeek et al., 2006; Kumar et al., 2016; Murphy et al., 2017; Lu et al., 2018). Given the large requirements for P by plants and microbes (Richardson et al., 2011), soil P availability should also directly or indirectly influence the RPE, but fewer studies have paid attention to the effect of P availability on the RPE compared with N availability (Dijkstra et al., 2013; Boilard et al., 2019; Xu et al., 2019). For example, high P availability increased the RPEs of two near-isogenic wheat lines, possibly due to larger biomass production, root exudation and microbial biomass (Xu et al., 2019). In another study, P fertilization increased the RPE of mutant barley lacking of root hairs, possibly because P fertilization exacerbated plant N deficiency in mutant barley thereby stimulating microbial SOM decomposition and mining for N (Boilard et al., 2019). These studies provide a basic understanding of P availability as a potential driver of the RPE and further imply that P fertilization may interact with soil N in influencing the RPE. The magnitude of RPE also depend on plant species and associated traits, such as root morphology (Pausch et al., 2016), root exudation (Dijkstra and Cheng, 2007), and associations with mycorrhizal fungi (Phillips and Fahey, 2006) and N_2-fixing bacteria (Zhu and Cheng, 2012). Legumes can fix N from the atmosphere through associations with rhizobia in root nodules. Legumes therefore may rely less on N, but more on P from soil. Grasses acquire N and P solely from soil either by releasing available substrates or by exploring soil volumes with developed fibrous root systems. Yet, how P availability affects the RPEs of legumes and grasses (*i.e.*, P availability and plant species interaction on the RPE) remains unclear.

The underlying mechanisms of RPE in response to nutrient availability are still elusive, where several hypotheses have been put forward (Cheng and Kuzyakov, 2005; Dijkstra et al., 2013). The plant-microbe competition hypothesis is usually proposed to explain a negative RPE when nutrient availability is extremely limited (Cheng, 1999). Under this condition, plant nutrient uptake strongly intensifies the competition for the same nutrients with microbes, which reduces microbial decomposition (Pausch et al., 2013; Yin et al., 2018). If nutrient limitation is alleviated by fertilization, the RPE will increase. The microbial nutrient mining hypothesis has been used to explain the positive RPE when nutrient availability is moderately low in soil (Fontaine et al., 2011). Under this condition, microbes could use root exudates to mine for nutrients from SOM to meet their nutrient demand (Kumar et al., 2016; Lu et al., 2019). When nutrient availability increases to a higher level, microbes mine less for nutrients from recalcitrant SOM and may prefer to use labile root exudates for their C and energy demand instead, causing a decrease in the RPE, which has also been termed as the preferential substrates utilization hypothesis (Kuzyakov and Cheng, 2004). These three hypotheses have been supported by observed RPEs associated with N availability (or N fertilization) in experimental studies (Blagodatskaya et al., 2007; Pausch et al., 2013; Kumar et al., 2016). Whether these nutrient-centered hypotheses are also responsible for explaining the RPE affected by P availability in conjunction with soil N needs to be further examined.

Here we examined the RPEs of ryegrass (*Lolium perenne* L., C_3 grass) and clover (*Trifolium repens* L., legume) with and without P fertilization at three sampling times (30, 44, and 58 days after planting) in an environmentally controlled growth chamber. A continuous ^{13}C–CO_2 labeling method was used to partition rhizosphere respiration and microbial respiration of SOM. A nutrient budget method was applied to evaluate the rhizosphere effect on net N and P release from soil for plant uptake. In this study, we hypothesized that: 1) clover would exhibit a smaller positive RPE than ryegrass because of its capacity for biological N fixation and thus a lower demand for N mined from SOM; 2) P fertilization would increase the RPE more in ryegrass than in clover because of a P-induced increase in N mining to meet increased plant N demand; 3) the RPE on SOC decomposition would be coupled with a plant-induced increase in net N mineralization, but not with a plant-induced increase in net P mobilization.

2. Materials and methods

2.1. Experimental design

The soil was collected from the top 15 cm in a grassland at John Bruce Pye Farm (33°55′51″ S, 150°39′38″ E) in Camden, NSW, Australia. The dominant species were the C_4 grasses *Paspalum dilatatum*, *Cyperus brevifolius* and *Setaria incrassata*, and the C_3 grass *Microlaena stipoides*. The soil was gently sieved through a 4 mm sieve to homogenize and remove most of the roots and large stones. The soil was a red-brown Chromosol according to the Australian Soil Classification (Isbell, 2002) with a pH of 5.4. The contents of sand, silt, and clay were 34%, 31%, and 35%, respectively. The concentrations of organic C, total N, and total P were 28.8, 2.5, and 0.15 mg g^{-1}, respectively. The concentrations of soil mineral N and extractable P were 58.0 and 8.7 mg kg^{-1}, respectively. The $\delta^{13}C$ value of this soil was −23.06‰.

Thirty-two bottom-capped polyvinyl chloride (PVC) pots (diameter 15 cm, height 20 cm) were filled with the grassland soil (equivalent to 3.20 kg oven-dried soil) at a bulk density of 0.91 g cm^{-3}. Each pot was equipped with a plastic tube for aeration and CO_2 trapping at the bottom, where the inside tube was attached with a small sponge and covered by a sandbag to prevent fine particles blocking the tube. After filling, soil moisture content was adjusted to 70% water holding capacity (21% gravimetric soil moisture content). Soils were amended with modified Hoagland nutrient solution containing macro- and micronutrients (N 10, K 15.2, S 5.8, Ca 2, Mg 2, B 0.01, Zn 0.05, Cu 0.01, Fe 0.05, and Mn 0.08 g m^{-2}), either with P or without P. Treatments with P were amended with KH_2PO_4 and K_2HPO_4 solution (4 g P m^{-2}, with a ratio of KH_2PO_4 and K_2HPO_4 adjusted to obtain a solution similar to the soil pH). Treatments without P were amended with the same amount of deionized water. Eight pots (4 pots with P and 4 pots without P) were destructively sampled to measure initial soil mineral N and extractable P one day after fertilization. Two days after fertilization, the remaining 24 pots were planted with either *Lolium perenne* L. (ryegrass), *Trifolium repens* L. (clover) or left unplanted (control). The six treatments were replicated 4 times. After germination, ryegrass and clover were thinned to 20 plants per pot.

The experiment was conducted in a controlled environment facility at the Centre for Carbon, Water and Food, The University of Sydney, Camden (NSW). During plant growth, the air temperature inside the growth chamber was kept at 25 °C from 6 p.m. to 6 a.m., and at 15 °C from 6 a.m. to 6 p.m. The relative air humidity was kept at 60%, and artificial lighting (Heliospectra, LX602C, 600 W) went on between 6 p.m. and 6 a.m. The CO_2 concentration was set at 800 ppm by injecting ^{13}C-depleted CO_2 into the chamber. This was needed to reduce the $\delta^{13}C$ value of CO_2 to a desired level, which would also promote plants to grow faster. The $\delta^{13}C$ value of CO_2 was −20 ± 0.3‰ (mean ± standard deviation) throughout the experiment (measured on a G2131-i Analyzer, Picarro, Santa Clara, CA, USA). This enabled us to effectively separate root-derived CO_2 and soil-derived CO_2. All pots were placed randomly in the growth chamber and watered every two days to maintain soil moisture content at 70% water holding capacity. Pots were randomly moved once a week to eliminate potential light effect. Pots were watered when lights were off to avoid plant photosynthesis of CO_2 respired by people entering the growth chamber.

2.2. Measurements

Total belowground CO_2 was measured 30, 44, and 58 days after planting using a dynamic chamber method (Yin et al., 2019). Briefly, at each sampling time, shoots were clipped to 1 cm above the soil surface, and then opaque PVC chambers with a septum (for collecting gas samples) and an outlet tube were sealed to the top of planted and unplanted pots with Blue-Tack (Bostik, Thomastown, Australia). Shoots were clipped so that the respiration measurements did not include shoot respiration. To circulate the gas phase of each pot individually, an aquarium pump was connected to the inlet tube at the bottom of the pot and the outlet tube of the chamber. Most of the initial CO_2 inside the chamber and pot was removed before gas sampling by attaching a column of soda lime to the circulation system for 1 h. Then the column of soda lime was removed from the circulation system (but air circulation was maintained), and a 12 ml gas sample was immediately taken from the septum of each chamber by a syringe (T_0). After 1 h (T_1) and 2 h (T_2), other 12 ml gas samples were taken. We acknowledge that stripping initial CO_2 from the system may disturb the aqueous carbonate equilibrium in the soil, which may cause retention of the subsequent CO_2 produced in the soil for reestablishing the equilibrium. This could affect respiration measurements in neutral or alkaline soil (Martens, 1987), but had probably minor effects in the slightly acidic soil we used here. All gas samples (T_0, T_1, and T_2) were transferred to pre-evacuated vials (Exetainers, Labco, UK). The CO_2 concentration and $\delta^{13}C$ of each gas sample were analyzed on a Delta V advantage isotope ratio mass spectrometer (IRMS) coupled to a Conflo IV and Flash HT (Thermo Fisher Scientific, Bremen, Germany).

After gas sampling on day 58, all pots were destructively harvested. The roots were carefully hand-picked from the soil. The clipped shoots from each sampling time and hand-picked roots after 58 days were washed, oven-dried at 60 °C, and grounded in a ball mill. The C%, N%, $\delta^{13}C$, and $\delta^{15}N$ of plant samples were analyzed on the IRMS. The P concentrations of plant samples were analyzed by ashing 0.5 g plant material at 550 °C for 4 h in a muffle furnace, digesting with 5 ml of 6 N HCl on a hotplate, and measuring the absorbance on the UV-VIS spectrophotometer (UVmini-1240) at 400 nm wavelength after adding ammonium molybdate-vanadate as the coloring reagent (Jackson, 1958). The fresh soil was homogenized and a representative soil sample (300 g) was taken for each pot. Fine roots were carefully removed from soil samples in planted treatments. Then these soils were prepared for measuring soil moisture content, mineral N, extractable P, microbial biomass C, N, and P. The oven-dried soils were grounded for measuring C%, N%, $\delta^{13}C$, and $\delta^{15}N$.

Mineral N (NH_4^+ and NO_3^-) was measured by extracting 5 g fresh soil sample with 40 ml 1 M KCl solution and filtering through Whatman No. 42 filter paper. The NH_4^+ and NO_3^- concentrations of the extracts were analyzed on a flow injection analyzer (FIA automated ion analyzer, Lachat Instruments, Loveland, CO, USA). Extractable P was measured by extracting 3 g fresh soil sample in 20 ml 0.03 N ammonium fluoride (NH_4F) with 0.025 N hydrochloric acid (HCl) and filtering through Whatman No. 42 filter paper. The P concentrations of the extracts were analyzed colorimetrically with ammonium molybdate-stannous chloride as the coloring reagent on the UV-VIS spectrophotometer (UVmini-1240) at 660 nm wavelength (Olsen and Sommers, 1982).

Microbial biomass C (MBC) and N (MBN) were measured using the chloroform fumigation- K_2SO_4 extraction method (Vance et al., 1987). One 15 g fresh soil sample was extracted with 40 ml 0.05 M K_2SO_4 solution, while another 15 g fresh soil sample was fumigated by ethanol-free chloroform in a vacuum desiccator in the dark for 48 h and then extracted with the same solution. Total organic C and total N of the extracts were analyzed on a TOC/TN analyzer (Shimadzu, TOC-Vcsh, TNM-1, Kyoto, Japan). MBC and MBN were calculated as the difference in total organic C and total N between the fumigated and non-fumigated soils adjusted by conversion factors of 0.45 and 0.54, respectively (Brookes et al., 1985). The remaining extracts were oven-dried at 60 °C, grounded, and measured for $\delta^{13}C$ on the IRMS. Microbial biomass P (MBP) was measured using the chloroform fumigation–NH_4F–HCl extraction method (Brookes et al., 1982). Fresh soil sample (3 g) was extracted in 20 ml 0.03 N NH_4F with 0.025 N HCl, and another fresh soil sample (3 g) was fumigated by ethanol-free chloroform in a vacuum desiccator in the dark for 24 h and then extracted as the non-fumigated sample. The P concentrations of the extracts were measured following the procedure for soil extractable P as described above. MBP was calculated as the difference in extractable P between fumigated and non-fumigated samples adjusted by a conversion factor of 0.4 (Brookes et al., 1982).

2.3. Calculations

2.3.1. Root-derived CO_2, soil-derived CO_2, and RPE

Total soil respiration (in mg CO_2–C kg^{-1} soil h^{-1}) in each pot was calculated as the slope of linear regression using the three CO_2 concentrations measured at T0, T1, and T2, with accounting for the air volume of the chamber and pot, the temperature, and the dried soil weight. The $\delta^{13}C$ value of corresponding total soil respiration was calculated based on the Keeling plot method where isotope ratios are plotted against the inverse of CO_2 concentrations (Pataki et al., 2003).

Total soil respiration in each planted pot was separated into soil-derived CO_2 (microbial decomposition of SOM) and root-derived CO_2 (root respiration and rhizosphere microbial respiration of labile substrates released by roots) using a two-source mixing model (Pausch et al., 2013):

$$C_S = C_T \times (\delta^{13}C_R - \delta^{13}C_T)/(\delta^{13}C_R - \delta^{13}C_S) \quad (1)$$

$$C_R = C_T - C_S \quad (2)$$

where C_T, C_S, and C_R are total belowground CO_2, soil-derived CO_2, and root-derived CO_2 in planted pots, respectively. $\delta^{13}C_T$ is the measured $\delta^{13}C$ value of total belowground CO_2 in planted pots, and $\delta^{13}C_S$ is the mean $\delta^{13}C$ value of soil respiration in unplanted control pots. We assumed that $\delta^{13}C$ values of soil-derived CO_2 in planted pots were the same as that in unplanted pots. Because rhizosphere priming may promote the decomposition of specific SOM with varying $\delta^{13}C$, the $\delta^{13}C$ of soil-derived CO_2 in planted pots may slightly differ from $\delta^{13}C_S$, but we expect this difference to be small compared to the difference between $\delta^{13}C_S$ and $\delta^{13}C_R$. $\delta^{13}C_R$ is the $\delta^{13}C$ value of root-derived CO_2 in planted treatments, which was calculated based on the $\delta^{13}C$ value of root tissue corrected by a fractionation factor of root-derived CO_2 relative to root tissue (−1.74‰ for grass and −2.67‰ for legume, averaged from cited papers, Werth and Kuzyakov, 2010).

The RPE was calculated as the difference in soil-derived CO_2 between planted treatments and unplanted control:

$$\mathrm{RPE} = \mathrm{CS\ (planted)} - \mathrm{CS\ (unplanted)} \quad (3)$$

2.3.2. Root-derived MBC and soil-derived MBC

Total MBC (MB_{Total}) in each planted pot was separated into soil-derived MBC (MB_{Soil}) and root-derived MBC (MB_{Root}) using a two-source mixing model (Shahzad et al., 2015):

$$MB_{Soil} = MB_{Total} \times (\delta^{13}C_{Root} - \delta^{13}C_{Total})/(\delta^{13}C_{Root} - \delta^{13}C_{Soil}) \quad (4)$$

$$MB_{Root} = MB_{Total} - MB_{Soil} \quad (5)$$

where $\delta^{13}C_{Root}$ is the $\delta^{13}C$ value of root tissue in planted pots, $\delta^{13}C_{Soil}$ is the mean $\delta^{13}C$ value of soil in unplanted control pots, and $\delta^{13}C_{Total}$ is the $\delta^{13}C$ value of total MBC in planted pots. The latter was calculated using the following equation:

$$\delta^{13}C_{Total} = (C_f \times \delta^{13}C_f - C_{nf} \times \delta^{13}C_{nf})/(C_f - C_{nf}) \quad (6)$$

where C_f and C_{nf} are the total organic C concentrations of fumigated and

non-fumigated extracts in planted pots, respectively, and $\delta^{13}C_f$ and $\delta^{13}C_{nf}$ are the $\delta^{13}C$ values of C_f and C_{nf}, respectively.

2.3.3. Net soil N and P release for plant uptake

Net soil N and P release for plant uptake in each pot were calculated after destructive harvest based on plant N and P content, biologically fixed N from the atmosphere in clover, soil mineral N and extractable P at the end of the experiment, and soil mineral N and extractable P at the start of the experiment. Net soil N release ($N_{release}$) was calculated as:

$$N_{release} = N_{plant} - N_{fix} + N_{\min,end} - N_{\min,start} \quad (7)$$

where N_{plant} is the N content in the total plant biomass (roots and shoots) after day 58 plus the N content in shoot biomass clipped on day 30 and 44, N_{fix} is the biologically fixed N in clover, and $N_{min,\ end}$ and $N_{min,\ start}$ are the soil mineral N measured at the end and start of the experiment, respectively.

Biologically fixed N from the atmosphere (N_{fix}) in clover treatments was calculated using the ^{15}N natural abundance approach (Mia et al., 2018):

$$N_{fix} = N_{clover} \times \left(\delta^{15}N_{ryegrass} - \delta^{15}N_{clover}\right) / \left(\delta^{15}N_{ryegrass} - \delta^{15}N_{bnf}\right) \quad (8)$$

where N_{clover} is the amount of total N in clover tissues, including clipped shoots at each sampling date and roots after day 58, $\delta^{15}N_{clover}$ is the weighted $\delta^{15}N$ value of clover tissues, $\delta^{15}N_{ryegrass}$ is the weighted $\delta^{15}N$ value of ryegrass tissues, including clipped shoots at each sampling date and roots after day 58 (used as a reference plant), and $\delta^{15}N_{bnf}$ is the $\delta^{15}N$ value of N-fixing plants completely relying on biological N fixation (without N uptake from soil), which was estimated as −1.527‰ for clover (Mia et al., 2018).

Net soil P release ($P_{release}$) was calculated as:

$$P_{release} = P_{plant} + P_{extr,end} - P_{extr,start} \quad (9)$$

where P_{plant} is the P content in the total plant biomass (roots and shoots) after day 58 plus the P content in shoot biomass clipped on day 30 and 44, and $P_{extr,\ end}$ and $P_{extr,\ start}$ are the soil extractable P measured at the end and start of the experiment, respectively.

For the planted treatments, "excess net soil N and P release" was also calculated by subtracting the average total amount of net soil N and P release in unplanted control from the total amount of net soil N and P release in planted treatments under each P fertilization level.

2.4. Statistical analyses

Repeated measures ANOVA analysis was used to test for the main effects of plant treatments (control, ryegrass, and clover) and P fertilization (with and without P fertilization), and their interaction, on soil-derived CO_2, and to test for the main effect of plant species (ryegrass and clover) and P fertilization, and their interaction, on root-derived CO_2 and the RPE. The repeated measures ANOVA included a random effect of date (Day). Two-way ANOVA analysis was used to test for the main and interactive effects of plant treatments (or species) and P fertilization on soil-derived CO_2, root-derived CO_2, and the RPE at each sampling time. In addition, two-way ANOVA analysis was also used to test for the main effects of plant species and P fertilization, and their interaction, on plant biomass, plant N and P content (for clover this includes biologically fixed N), plant N acquisition from soil, and root-derived MBC, and to test for the main effects of plant treatments and P fertilization, and their interaction, on soil mineral N, soil extractable P, and net soil N and P release for plant uptake. *Post hoc* Tukey's HSD tests were used to compare the differences among means. Pearson correlation analysis and simple linear regression were used to relate the RPE with excess net soil N and P release for plant uptake. All statistical analyses were done with SPSS 20.0. The significance level was set at $p < 0.05$.

3. Results

3.1. Plant biomass, plant N and P content and acquisition from soil

Shoot biomass was significantly affected by plant species and P fertilization at each sampling time (Tables 1 and 3). Ryegrass had larger shoot biomass than clover by Day 30, while clover had larger shoot biomass than ryegrass by Day 44 and Day 58 (Table 1). The clipped shoot biomass accounted for 24%, 18%, and 22% of total biomass in ryegrass and accounted for 8%, 29%, and 41% of total biomass in clover, respectively (Table 1). Clover showed a larger production of total shoot biomass and total plant biomass during the experiment, but showed smaller root biomass and ratio of root to shoot than ryegrass (Table 1). P fertilization significantly increased shoot biomass of both species by Day 30, but decreased shoot biomass of ryegrass and did not affect shoot biomass of clover by Day 44 and Day 58 (Table 1). P fertilization had no significant effect on total shoot, root biomass, total biomass, and the ratio of root to shoot of both species (Tables 1 and 3).

Plant N and P content exhibited a similar pattern as plant biomass (Tables 2 and 3). Ryegrass showed larger shoot N and P content than clover by Day 30, while clover showed larger shoot N and P content than ryegrass by Day 44 and Day 58 (Table 2). Although having smaller root biomass, clover showed larger root N content than ryegrass, possibly due to biological N fixation by rhizobia in root nodules of clover. In the clover treatment, biologically fixed N from the atmosphere accounted for about 37% of plant N content on average (Fig. 1A). Excluding biologically fixed N, clover still showed larger plant N acquisition from soil compared to ryegrass (Fig. 1A). P fertilization significantly increased shoot N content of both species by Day 30, whereas by Day 44 and Day 58, P fertilization significantly decreased shoot N content of ryegrass, and did not affect shoot N content of clover (Table 2). P fertilization significantly increased shoot P content of both species by Day 30 and of clover by Day 44, but did not affect shoot P content of ryegrass by Day 44 and Day 58 (Table 2). When combined over the whole growth period, P fertilization significantly decreased plant N acquisition from soil, but increased plant P content (Figs. 1A and 2A).

3.2. Root-derived CO_2, soil-derived CO_2, and RPE

Root-derived CO_2 was significantly affected by plant species, P fertilization, sampling time, and their interactions (Fig. 3A). Ryegrass exhibited larger root-derived CO_2 than clover by Day 30, while clover exhibited larger root-derived CO_2 than ryegrass by Day 44 and Day 58, similar to their shoot biomass (Table 4 and Fig. 3A), but despite a smaller root biomass compared to ryegrass by Day 58 (Table 1). P fertilization significantly increased root-derived CO_2 of both species by Day 30. However, P fertilization significantly decreased root-derived CO_2 of ryegrass by Day 44 and Day 58 (Table 4 and Fig. 3A), which aligned well with shoot biomass and shoot N content in response to P fertilization (Tables 1 and 2).

RPE was significantly affected by plant species, sampling time and their interaction, and by P fertilization (Fig. 3B). Both species caused larger soil-derived CO_2 than the unplanted control (Table S1), indicating positive RPEs, except for the clover treatment without P fertilization by Day 30 (Fig. 3B). Ryegrass exhibited a larger RPE than clover by Day 30 (41% vs. 1%), while clover exhibited a larger RPE than ryegrass by Day 44 (51% vs. 22%) and Day 58 (117% vs. 13%) (Table S1 and Fig. 3B). P fertilization did not significantly affect the RPE by Day 30 and the RPE of clover by Day 44 and Day 58, but significantly decreased the RPE of ryegrass by Day 44 and Day 58 (Table 4 and Fig. 3B).

3.3. Microbial biomass C (root- and soil-derived MBC), microbial biomass N, and microbial biomass P

Total MBC was significantly greater in planted treatments, and soil-derived MBC was significantly greater in clover than in the unplanted

Table 1
Shoot, root, and total biomass, and root-to-shoot ratio of ryegrass and clover with and without P fertilization (T1, Day 30; T2, Day44; T3, Day 58). Values are shown as mean ± SE (n = 4).

Treatments	T1-shoot (g pot^{-1})	T2-shoot (g pot^{-1})	T3-shoot (g pot^{-1})	Total shoot (g pot^{-1})	Root (g pot^{-1})	Total (g pot^{-1})	Root/Shoot
Ryegrass - P	2.44 ± 0.12b	2.85 ± 0.26b	3.81 ± 0.21b	9.10 ± 0.32b	5.13 ± 0.34 ab	14.22 ± 0.46b	0.57 ± 0.04a
Ryegrass + P	4.53 ± 0.07a	2.26 ± 0.03c	2.60 ± 0.19c	9.39 ± 0.15b	5.34 ± 0.37a	14.73 ± 0.45b	0.57 ± 0.04a
Clover - P	1.06 ± 0.08d	5.38 ± 0.07a	8.02 ± 0.44a	14.46 ± 0.54a	4.52 ± 0.19 ab	18.98 ± 0.73a	0.31 ± 0.00b
Clover + P	1.74 ± 0.15c	5.42 ± 0.03a	7.48 ± 0.29a	14.65 ± 0.21a	4.03 ± 0.25b	18.68 ± 0.47a	0.27 ± 0.01b

Table 2
Plant N and P content in shoot and root biomass of ryegrass and clover with and without P fertilization (T1, Day 30; T2, Day44; T3, Day 58). Values are shown as mean ± SE (n = 4).

Treatments	Plant N				Plant P			
	T1-shoot N (mg pot^{-1})	T2-shoot N (mg pot^{-1})	T3-shoot N (mg pot^{-1})	Root N (mg pot^{-1})	T1-shoot P (mg pot^{-1})	T2-shoot P (mg pot^{-1})	T3-shoot P (mg pot^{-1})	Root P (mg pot^{-1})
Ryegrass - P	65.6 ± 1.8b	58.4 ± 5.0b	57.5 ± 5.0b	59.3 ± 3.4b	2.66 ± 0.15b	3.72 ± 0.28c	6.31 ± 0.28b	5.13 ± 0.30a
Ryegrass + P	101.4 ± 4.3a	28.6 ± 1.8c	31.5 ± 1.2b	52.3 ± 4.1b	4.96 ± 0.17a	3.70 ± 0.09c	6.44 ± 0.47b	4.29 ± 0.20a
Clover - P	30.2 ± 1.8c	101.0 ± 1.2a	194.2 ± 9.7a	132.9 ± 8.2a	1.22 ± 0.16c	6.69 ± 0.27b	14.67 ± 0.98a	4.43 ± 0.21a
Clover + P	54.5 ± 3.6b	98.3 ± 1.8a	188.1 ± 7.2a	126.4 ± 11.4a	2.01 ± 0.17b	8.19 ± 0.24a	14.83 ± 0.35a	4.97 ± 0.38a

control, while root-derived MBC was similar between ryegrass and clover treatments (Fig. 4). P fertilization substantially increased total MBC across the two species (Fig. 4A), mainly due to a significant increase of root-derived MBC with P fertilization (Fig. 4C).

The MBN was similar between ryegrass and clover treatments and higher in both species than in the unplanted control (Table 5). The MBP did not significantly differ among the two species and the unplanted treatments (Tables 4 and 5). P fertilization significantly increased MBN in ryegrass and clover treatments, but did not affect MBN in the unplanted treatment (Table 5). P fertilization marginally increased MBP across the two species and the unplanted treatments (Tables 4 and 5).

3.4. Mineral N, extractable P, net soil N and P release

Soil mineral N and extractable P were significantly lower in planted treatments than in the unplanted control, possibly due to large plant N and P acquisition from soil in ryegrass and clover (Figs. 1 and 2). Although clover exhibited larger plant N and P acquisition from soil than ryegrass, soil mineral N and extractable P did not differ between the two species (Figs. 1B and 2B). P fertilization significantly increased soil extractable P (Fig. 2B), but did not significantly affect soil mineral N (Fig. 1B).

Net soil N release was larger in planted treatments than in the unplanted control, except for the ryegrass treatment with P fertilization (Fig. 1C). Clover caused a larger net soil N release than ryegrass (Fig. 1C). P fertilization significantly decreased net soil N release in ryegrass and clover treatments, but did not significantly affect net soil N release in the unplanted control (Fig. 1C).

Net soil P release was negative in the unplanted control, suggesting net P immobilization, while net soil P release (or net P mobilization) was positive in planted treatments, except for the ryegrass treatment with P fertilization (Fig. 2C). Clover caused a larger net P mobilization than ryegrass, especially without P fertilization (Fig. 2C). P fertilization significantly caused more net P immobilization in the unplanted control, changed net P mobilization to net P immobilization in ryegrass treatment, and decreased net P mobilization in clover treatment (Fig. 2C).

3.5. Correlations between the RPE and excess net soil N and P release

When relating excess net soil N and P release (net soil N or P release in planted treatments minus average net soil N or P release in the unplanted control) to cumulative RPE, we did not observe significant linear relationships (R^2 = 0.067, p = 0.334; R^2 = 0.007, p = 0.760, respectively, data not shown). However, significant and positive relationships were found between excess net soil N release and the RPE by Day 58 ($R^2 = 0.742$, $p < 0.001$, Fig. 5A), and between excess net soil P release and the RPE by Day 58 ($R^2 = 0.505$, $p = 0.002$, Fig. 5B) across all planted treatments.

4. Discussion

4.1. Species effects on RPE

Consistent with other studies on grassland species (Shahzad et al., 2012; Nie and Pendall, 2016; Murphy et al., 2017; Lu et al., 2019), both ryegrass and clover induced positive RPEs in most plant-soil combinations, with the magnitude ranging from −1%–134% compared to the unplanted soil. However, the greater RPE occurred earlier with ryegrass compared to clover (Fig. 3B). These results suggest that interspecific differences in the RPE on SOM decomposition depends on plant phenology (Cheng et al., 2003; Pausch et al., 2013). Initially, ryegrass grew faster than clover (as suggested by the larger shoot biomass of ryegrass during the first 30 days, Table 1), and may have allocated more C to the rhizosphere (as suggested by the larger root-derived CO_2 of ryegrass by Day 30, an indicator for root activity and quantity of rhizodeposits, Fig. 3A). The greater C allocation belowground may have promoted soil C decomposition thereby causing a larger RPE (Fig. 3B). In contrast, at later stages, the regrowth of clover was faster than that of ryegrass (Table 1) and allocated more C to the rhizosphere (Fig. 3A), possibly because of higher C and energy demand for biological N fixation by rhizobia associated with legumes. This may also have accelerated soil C decomposition and induced a larger RPE (Fig. 3B). Therefore, the different RPEs of ryegrass and clover at different stages were likely due to distinct aboveground biomass regrowth rates, root activity, and quantity of rhizodeposits. Previous studies also suggested that the RPE on SOC decomposition was tightly related to plant biomass and quantity of rhizodeposits (Dijkstra et al., 2006; Bengtson et al., 2012; Wang et al., 2016). Nevertheless, the observation of a larger RPE in clover than in ryegrass during later stages was in contrast to what we hypothesized. We expected smaller RPEs in clover than in ryegrass because of a lower demand for soil N. Therefore, clover would need less rhizodeposition for enhancing SOM decomposition and N release, and instead could spend more C to support biological N fixation from the atmosphere. Although clover received about 37% of its N from biological N fixation, it also took up more N and P from the soil than ryegrass by the end of the experiment (Figs. 1A and 2A), possibly through a larger RPE. It has also been suggested that a higher substrate quality of rhizodeposits can increase the RPE by legumes as compared to non-legumes (Cheng et al., 2003; Zhu

Table 3
Two-way ANOVA p-values for plant biomass, plant N and P content (T1, Day 30; T2, Day44; T3, Day 58).

	T1-shoot biomass	T2-shoot biomass	T3-shoot biomass	Shoot biomass	Root biomass	Total biomass	Root/ Shoot	T1-shoot N	T2-shoot N	T3-shoot N	Root N	T1-shoot P	T2-shoot P	T3-shoot P	Root P
Species	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.007**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.976
P	**<0.001**	0.066	**0.012**	0.497	0.659	0.849	0.594	**<0.001**	**<0.001**	**0.031**	0.382	**<0.001**	**0.008**	0.810	0.605
Species × P	**<0.001**	**0.039**	0.276	0.877	0.262	0.469	0.540	0.084	**0.001**	0.157	0.974	**0.001**	**0.006**	0.987	**0.029**

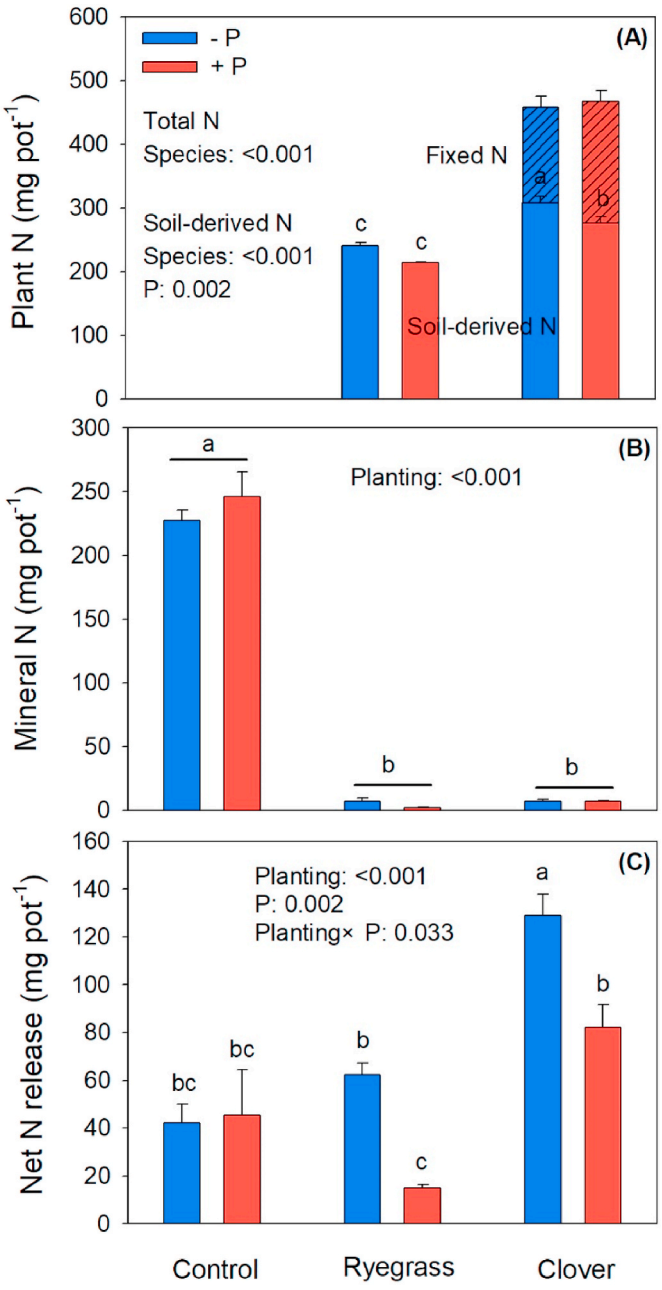

Fig. 1. Plant N content (A), soil mineral N (B), and net soil N release (C) for unplanted, ryegrass, and clover treatments with and without P fertilization at the end of the experiment. For clover, plant N content is further separated into plant N acquisition from the soil ("Soil-derived N″) and biologically fixed N ("Fixed N″, shaded bars). Sub-legend shows ANOVA p-values. Different letters indicate significant differences among treatments. Error bar indicates one standard error of the mean.

and Cheng, 2012; Drake et al., 2013).

The decreasing RPE of ryegrass with time may have to do with gradually limited soil N availability. Although we did not measure soil available N after each shoot harvest, it could be inferred that the regrowth of ryegrass was N limited from the decrease of shoot biomass and shoot N content of ryegrass regrowth at later stages compared to that at early stage, particularly with P fertilization (Tables 1 and 2). In contrast, the regrowth of clover may be less affected by soil N limitation because of the potential for biological N fixation from the atmosphere, as suggested by the consistent increase of shoot biomass and shoot N content of clover regrowth with time (Tables 1 and 2). These results are

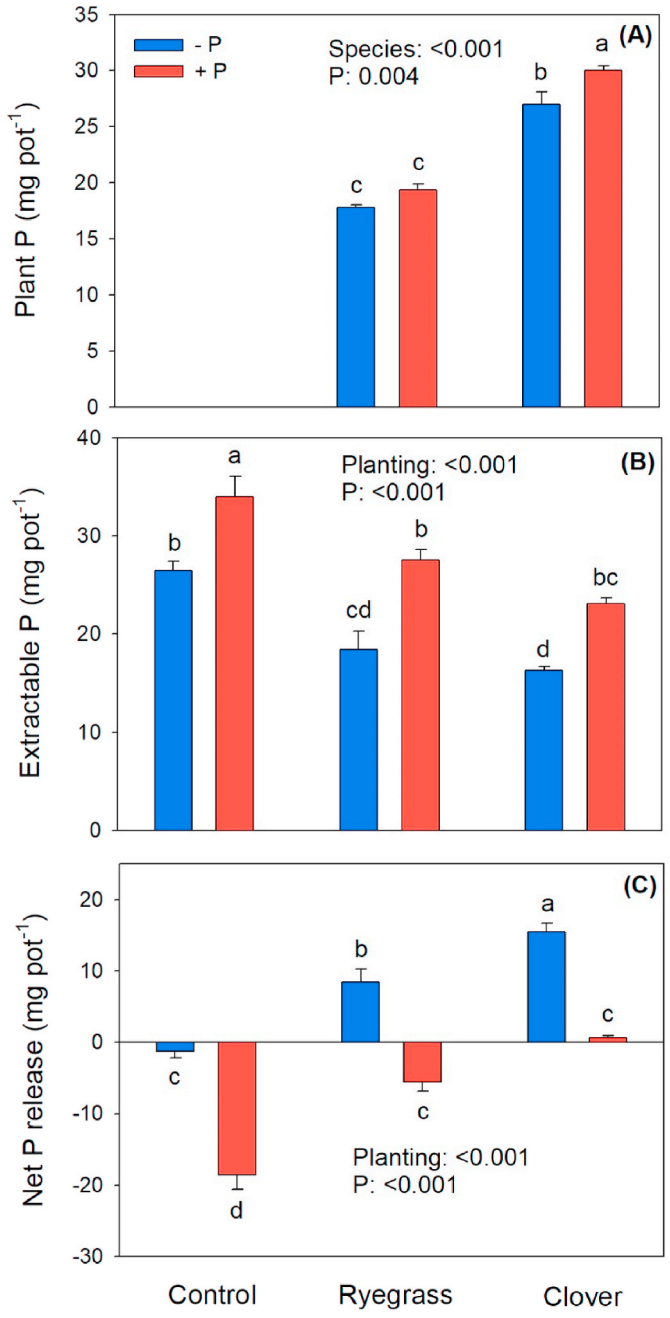

Fig. 2. Plant P content (A), soil extractable P (B), and net soil P release (C) for unplanted, ryegrass, and clover treatments with and without P fertilization at the end of the experiment. Sub-legend shows ANOVA p-values. Different letters indicate significant differences among treatments. Error bar indicates one standard error of the mean.

somewhat inconsistent with earlier studies on annual crops, perennial grassland species, or trees (Cheng et al., 2003; Dijkstra and Cheng, 2007; Lu et al., 2019), where the RPEs increased continuously with sampling time until flowering stage or until the end of the experiment. Possibly, clipping to 1 cm height before measuring soil respiration caused soil N limitation with a large growth reduction, which in turn influenced microbial decomposition of SOM and hence the distinct responses of RPE to sampling time of these grassland species.

4.2. P effect on RPE and interaction with species

In contrast to our second hypothesis, P fertilization decreased the

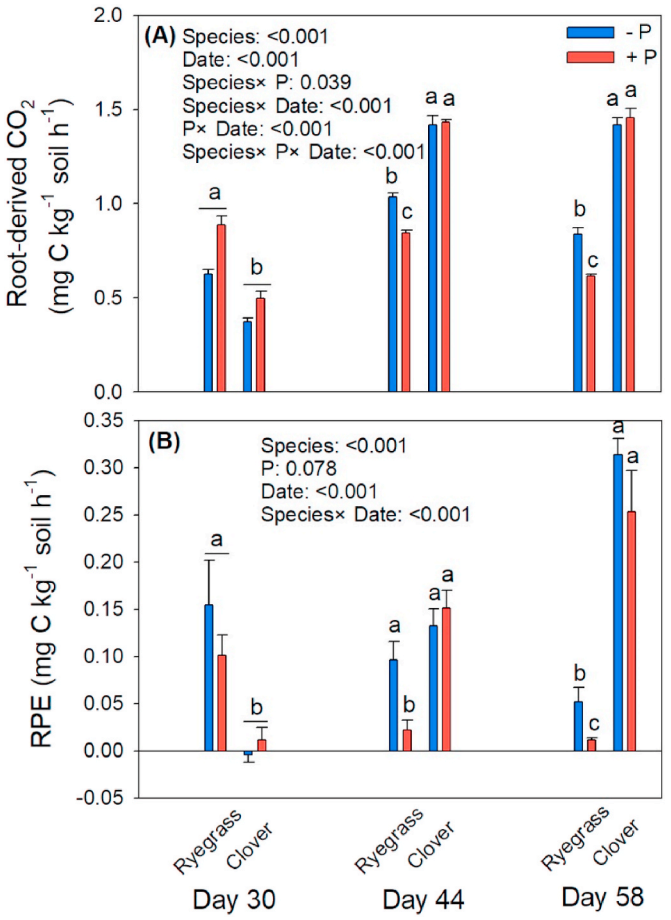

Fig. 3. Root-derived CO_2 (A) and RPE (B) under ryegrass and clover treatments with and without P fertilization 30, 44, and 58 days after planting. Sub-legend shows ANOVA p-values. Different letters indicate significant differences among treatments at each sampling time. Error bar indicates standard error of the mean.

RPE of ryegrass at later stages, but not that of clover, suggesting that P fertilization and plant species interactively affected the magnitude of RPE. Initially, P fertilization did not affect the RPEs of both species, possibly because plants were small and soil available N and P were relatively high. At later stages, there was still some extractable P left in the planted soil, but mineral N was depleted (Figs. 1B and 2B). Under this circumstance, P fertilization substantially enhanced microbial biomass N in both ryegrass and clover treatments (Table 5), and therefore more N immobilization may have occurred under P fertilization when there was C supply from root exudates. The P-induced N limitation due to microbial N immobilization may have intensified competition for N with plant roots. This competition may have negatively impacted plant growth and root exudation of ryegrass (Tables 1 and 2, Fig. 3A), thereby decreasing the RPE (Figs. 3B and 6A). In contrast, the enhanced microbial N immobilization did not negatively impact clover as much. Possibly, biological N fixation by clover alleviated soil N limitation that would otherwise constrain plant growth and root exudation (Fig. 6B). Indeed, P fertilization enhanced the percentage of biologically fixed N in clover when plant N demand from soil was limited (Fig. 1A). Furthermore, ryegrass exhibited less net soil N release, while clover still exhibited more net soil N release compared to the unplanted control with P fertilization (Fig. 1C), further indicating that P-induced microbial N immobilization with C supply affected ryegrass more than clover in terms of their rhizosphere effects on SOM decomposition and nutrient release.

However, these results were in contrast to previous studies where P

Table 4
Two-way ANOVA p-values for root-derived CO_2, RPE, microbial N, and microbial P (T1, Day 30; T2, Day44; T3, Day 58).

	T1-root-derived CO_2	T2-root-derived CO_2	T3-root-derived CO_2	T1-RPE	T2- RPE	T3- RPE	Microbial N	Microbial P
Species	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.146
P	**<0.001**	**0.008**	**0.024**	0.502	0.138	**0.002**	**<0.001**	**0.068**
Species × P	0.073	**0.003**	**0.003**	0.223	**0.020**	**0.014**	0.098	0.242

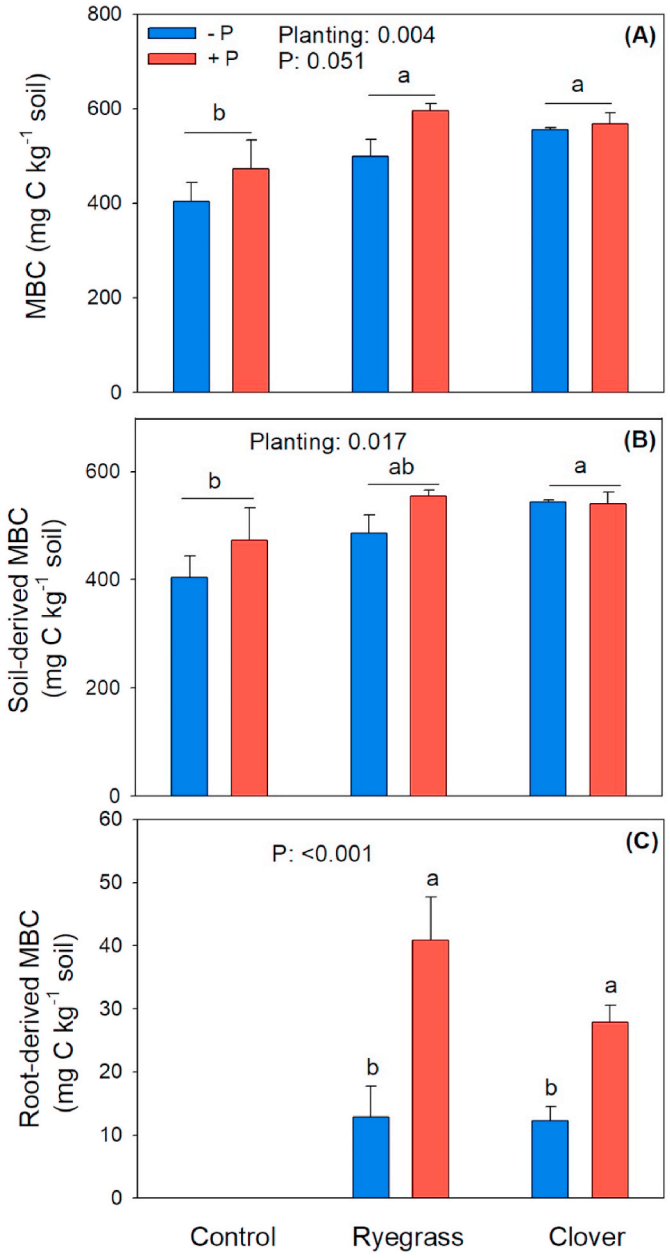

Fig. 4. Microbial biomass C (MBC, A), soil- (B) and root-derived MBC (C) under unplanted, ryegrass, and clover treatments with and without P fertilization at the end of the experiment. Sub-legend shows ANOVA p-values. Different letters indicate significant differences among treatments. Error bar indicates one standard error of the mean.

Table 5
Microbial N and P at the end, soil mineral N and extractable P at the start of the experiment in the soil of unplanted control, ryegrass, and clover treatments with and without P fertilization. Values are shown as mean ± SE (n = 4).

Treatment	Microbial N (mg N kg^{-1} soil)	Microbial P (mg P kg^{-1} soil)	Soil mineral N at the start (mg N kg^{-1} soil)	Soil extractable P at the start (mg P kg^{-1} soil)
Control - P	8.4 ± 0.9c	14.2 ± 1.9a	58.0 ± 1.8	8.7 ± 0.4
Control + P	9.6 ± 0.6c	14.3 ± 1.7a	62.8 ± 7.7	16.4 ± 1.1
Ryegrass - P	36.1 ± 2.4b	14.7 ± 2.2a	–	–
Ryegrass + P	42.0 ± 0.8a	20.4 ± 1.5a	–	–
Clover - P	35.9 ± 1.2b	15.3 ± 1.4a	–	–
Clover + P	42.3 ± 0.8a	17.1 ± 0.5a	–	–

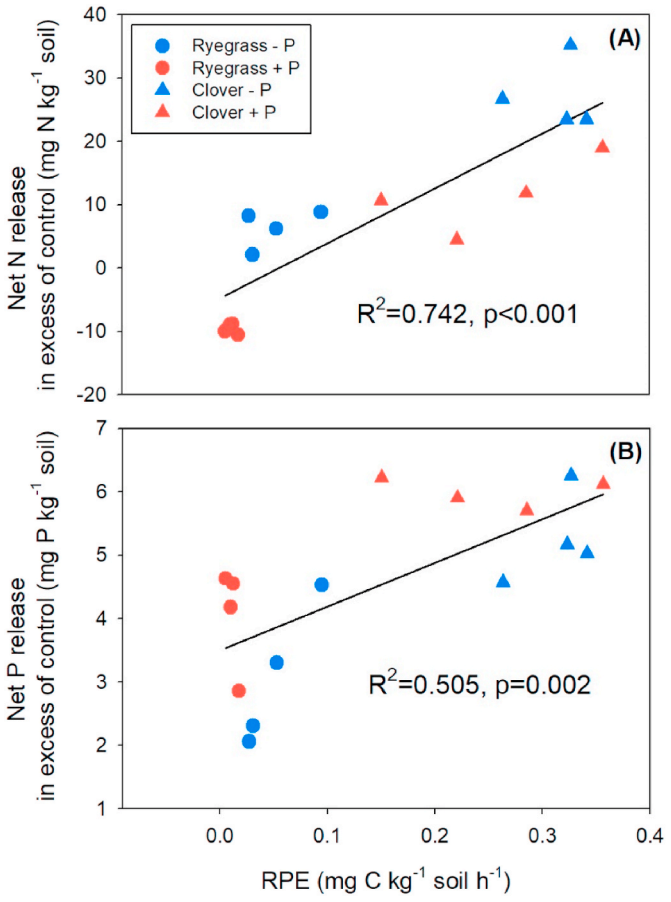

Fig. 5. Linear correlations between RPE 58 days after planting and net soil N (A) and P (B) release in excess of control during the entire experiment under ryegrass and clover treatments with and without P fertilization.

fertilization increased the RPEs of barley or wheat (Boilard et al., 2019; Xu et al., 2019). In these published studies, P fertilization similarly exacerbated soil N limitation, but also significantly promoted plant growth and root exudation which dominantly controlled the RPE (*e.g.*, Huo et al., 2017). In contrast, P fertilization in the present case reduced plant growth and root exudation of ryegrass at later stages, which resulted in the lower RPE. Possibly, the contrasting effect of P-fertilization on plant growth and root exudation between our study and the two published studies caused the opposite RPEs in response to P

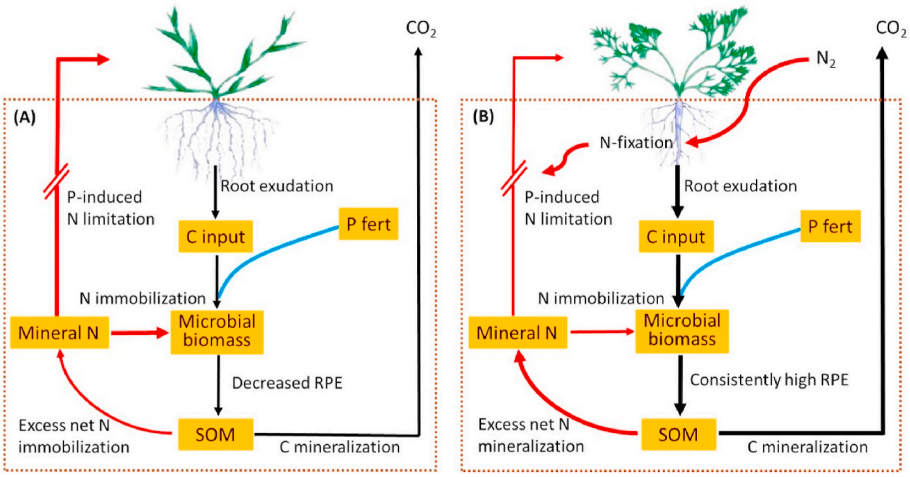

Fig. 6. A new framework showing C–N–P interactions on the RPE. Different colors are used to indicate the C (black), N (red) and P (blue) flows. The direction of each arrow indicates the logical follow and the relative thickness of each arrow-line indicates the relative strength of the linkage. Specifically, P fertilization coupled with C input from root exudates enhances microbial N immobilization from mineral N into microbial biomass, which reduces the RPE of non N-fixing grasses when soil N limitation negatively impacts plant growth and root exudation (A). In contrast, P-induced microbial N immobilization does not affect the RPE of N-fixing legumes because their biological N fixation alleviates N limitation for plant growth (B). As a result, net N immobilization and small RPEs predominate in grasses with P fertilization, while net N mineralization and relatively large RPEs predominate in legumes with P fertilization. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

fertilization. It is also possible that, in the published studies, soil available N was only moderately limited, so that root exudates could stimulate microbes to mine for N from SOM ("microbial N mining" mechanism). But, in our study, P-induced N limitation to ryegrass may have been more severe because of the frequent clipping resulting in a reduced RPE due to the constraints on plant growth and microbial activity ("competition for N between plants and microbes" mechanism). As ryegrass and clover are frequently used for grazing, it is expected that clipping shoot biomass would have similar effects to grazing. Plant clipping could cause (temporary) changes in belowground CO_2 fluxes (including root respiration, exudation and the RPE), but we are not aware if clipping effects would differ among species and P fertilizer treatments.

Consistent with the enhanced soil MBN, P fertilization also substantially promoted more root-derived C incorporation into MBC, suggesting that soil microbes preferred labile root-derived C to recalcitrant SOM-derived C as long as mineral nutrients (such as P) supply was abundant. Previous studies also showed that P fertilization could promote microbial growth rate when ample amounts of easily available C and N were added (Ehlers et al., 2010). As described above, P-induced microbial N immobilization may cause a strong competition for N between microbes and plants, which in turn negatively impacts plant growth, root exudation, and further SOM decomposition. On the other hand, P-induced microbial N immobilization may lead to more microbial biomass growth from labile root-derived C, and less extracellular enzyme production to decompose recalcitrant SOM-derived C, thereby decreasing the RPE. This is feasible given that there may be a trade-off between the formation of microbial biomass and the production of relatively N-costly extracellular enzymes (Sterner and Elser, 2002; Allison, 2005; Shahzad et al., 2015). When microbial N immobilization dominates with limited mineral N left in soil, C input from root exudates could further enhance microbial N immobilization from the mineral N pool. Consequently, P-fertilization indirectly regulates RPE by influencing microbial N mineralization and/or immobilization.

4.3. Relationships between RPE and net soil N and P release

The higher net soil P release in planted treatments compared to the unplanted control may be attributed to the exudation of organic acid anions (*e.g.*, citrate and malate). Leguminous species usually have a higher capacity for organic acid anions secretion to mobilize P from soil compared to non-leguminous species (*e.g.*, Nuruzzaman et al., 2006), which may have resulted in greater net P release for clover than for ryegrass (Fig. 2C). Importantly, organic acid anions could also break down organic-mineral associations and/or displace SOC on mineral surfaces (Kleber et al., 2015), thus leading to an increased priming effect (Keiluweit et al., 2015). This could explain the significant positive relationship between excess net P release and the RPE by Day 58 (Fig. 5B). However, the lower or lack of an increase of net N release in planted treatments compared to the unplanted control (Fig. 1C) suggests that in planted treatments increased microbial N immobilization occurred in parallel with increased N mineralization. Further, differences in excess net N release could in a large part be explained by the differences in the RPE by Day 58 across all plant-soil treatments (Fig. 5A). Several studies have also shown that increased net N mineralization by plants was positively correlated with RPE (Cheng, 2009; Dijkstra et al., 2009; Cheng, 2009; Zhu and Cheng, 2012), and microbial organic P mineralization was related to enhanced organic C decomposition (Achat et al., 2012; Spohn and Kuzyakov, 2013). Overall, our results suggest that the increase in net soil N and P release by plants can be directly linked with the magnitude of the RPE on SOM mineralization.

Cumulative RPE was not correlated with excess net soil N or P release across all plant-soil treatments during the entire experiment (data not shown), although positive RPE was observed at individual sampling times. Possibly, there was a time lag between the RPE and nutrient release made available to plants. Initially, the RPE may not have benefitted plants in terms of nutrient uptake, because nutrient availabilities were relatively high in this period and plant nutrient demand was perhaps not limited by rhizosphere priming effects on SOM decomposition. Instead, the N and P released from SOM via the RPE at an early stage may have either been retained in the increased microbial biomass or were not immediately taken up by plants. Only when soil nutrients (especially N) became more limited due to increased plant growth during later stages did the RPE benefit both microbes and plants in terms of N and P uptake. Due to the shorter life cycle of microbes (*i.e.*, higher microbial biomass turnover) compared to plant roots, the nutrients taken up by microbes will be eventually returned to the soil solution, and subsequently be captured by plants. Overall, our results support the emerging view that the RPE is an indirect co-evolved mutualism providing benefits to both plants and rhizosphere microbes (Cheng et al., 2014).

4.4. Implications for soil C, N and P dynamics

Our results suggest that SOC decomposition can be enhanced by the RPE of grassland species, and the magnitude of RPE can be directly linked to soil nutrient availability and plant nutrient acquisition. The rates of primed C decomposition were 0.03 and 0.28 mg C kg^{-1} soil h^{-1} in ryegrass and clover treatments by the end of the experiment,

respectively. Meanwhile, the excess net soil N release were −5.2 and 61.7 mg pot^{-1}, and the excess net soil P release were 11.4 and 18.0 mg pot^{-1} in ryegrass and clover treatments, respectively. Therefore, besides N, the increase in SOC decomposition induced by the RPE may also increase P mobilization and plant P acquisition. Further studies should focus more on how root exudates and rhizosphere microbes mediate soil P mobilization as well as soil C mineralization, which is crucial for better understanding biogeochemical processes in response to global climate change.

In conventional frameworks, decomposable organic C and mineral N are considered to be the most important soil factors in mediating the direction and magnitude of RPE (Kuzyakov, 2002). Moreover, the mechanisms of RPE can switch depending on the different amounts of decomposable organic C and mineral N (Kuzyakov, 2002; Dijkstra et al., 2013). Here, we put forward a new framework that C–N–P interactively affects the RPE: P fertilization coupled with C supply from root exudates could induce microbial N immobilization, thereby reducing the RPE of non N-fixing grasses when soil N limitation negatively affects plant growth and root exudation; P-induced microbial N immobilization does not affect the RPE of N-fixing legumes, however, because biological N fixation can alleviate soil N limitation and its negative effects on plant growth and root exudation (Fig. 6). These results demonstrate the importance of C–N–P interactions for understanding the RPE, and of equal significance is that the RPE is not only key to C and N but also to P cycling in plant-soil systems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.108005.

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