



Belowground Carbon Efficiency for Nitrogen and Phosphorus Acquisition Varies Between *Lolium perenne* and *Trifolium repens* and Depends on Phosphorus Fertilization

Jiayu Lu^{1,2}, Jinfeng Yang³, Claudia Keitel², Liming Yin¹, Peng Wang^{1*}, Weixin Cheng⁴ and Feike A. Dijkstra²

¹ CAS Key Laboratory of Forest Ecology and Management, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China, ² School of Life and Environmental Sciences, Sydney Institute of Agriculture, The University of Sydney, Sydney, NSW, Australia, ³ National Engineering Laboratory for Efficient Utilization of Soil and Fertilizer Resources, College of Land and Environment, Shenyang Agricultural University, Shenyang, China, ⁴ Environmental Studies Department, University of California, Santa Cruz, Santa Cruz, CA, United States

OPEN ACCESS

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*Correspondence:

Peng Wang
wangpeng@iae.ac.cn

Specialty section:

This article was submitted to
Plant Nutrition,
a section of the journal
Frontiers in Plant Science

Received: 24 April 2022

Accepted: 03 June 2022

Published: 24 June 2022

Citation:

Lu J, Yang J, Keitel C, Yin L,
Wang P, Cheng W and Dijkstra FA
(2022) Belowground Carbon
Efficiency for Nitrogen
and Phosphorus Acquisition Varies
Between *Lolium perenne* and *Trifolium
repens* and Depends on Phosphorus
Fertilization.
Front. Plant Sci. 13:927435.
doi: 10.3389/fpls.2022.927435

Photosynthetically derived carbon (C) is allocated belowground, allowing plants to obtain nutrients. However, less is known about the amount of nutrients acquired relative to the C allocated belowground, which is referred to as C efficiency for nutrient acquisition (CENA). Here, we examined how C efficiency for nitrogen (N) and phosphorus (P) acquisition varied between ryegrass (*Lolium perenne*) and clover (*Trifolium repens*) with and without P fertilization. A continuous ¹³C-labeling method was applied to track belowground C allocation. Both species allocated nearly half of belowground C to rhizosphere respiration (49%), followed by root biomass (37%), and rhizodeposition (14%). With regard to N and P, CENA was higher for clover than for ryegrass, which remained higher after accounting for relatively low C costs associated with biological N₂ fixation. Phosphorus fertilization increased the C efficiency for P acquisition but decreased the C efficiency for N acquisition. A higher CENA for N and P in clover may be attributed to the greater rhizosphere priming on soil organic matter decomposition. Increased P availability with P fertilization could induce lower C allocation for P uptake but exacerbate soil N limitation, thereby making N uptake less C efficient. Overall, our study revealed that species-specific belowground C allocation and nutrient uptake efficiency depend on which nutrient is limited.

Keywords: belowground carbon allocation, biological nitrogen fixation, carbon allocation for nutrient uptake, ¹³C-labeling, rhizosphere priming effect

INTRODUCTION

Belowground carbon (C) allocation by plants is an important driver for plant nutrient acquisition. In a global synthesis, Pausch and Kuzyakov (2018) reported that grassland species allocated on an average of 33% of gross primary productivity belowground to root biomass, rhizosphere respiration, and rhizodeposition. This belowground C is tightly associated with plant nutrient acquisition through various strategies, such as generating fine roots or root hairs, forming

symbiotic associations with nitrogen (N)-fixing bacteria or mycorrhizal fungi, or stimulating microbial activity to mobilize nutrients from soil organic matter using root exudates (Lambers et al., 2008; Richardson et al., 2009; Zhu et al., 2014). Modeling studies have indicated that variation in C allocation for nutrient acquisition between N-fixing and non-fixing plants, or between arbuscular and ectomycorrhizal plants, is helpful for understanding their competitive advantages and how this relates to their abundance and productivity (Fisher et al., 2010; Brzostek et al., 2014). However, empirical research on how much total C plants allocate belowground to obtain nutrients is rare.

To assess the C efficiency associated with nutrient acquisition, we introduce a new parameter, i.e., C efficiency for nutrient acquisition (CENA), which we define as the amount of nutrients acquired relative to C allocated belowground (Wang et al., 2022). In most studies, belowground C allocation is primarily based on measures of root production, thus ignoring other C pathways such as allocation to root respiration, root exudates, and symbiotic relationships, which are extremely difficult to quantify (Vicca et al., 2012; Pausch and Kuzyakov, 2018; Keller et al., 2021). This assessment, without considering other C pathways, may underestimate total belowground C allocation, hindering an accurate understanding of CENA. ^{13}C -labeling methods provide us with the opportunity to quantify root respiration, root exudates, and symbiotic microbial respiration, which have been successfully applied in previous studies (Schmitt et al., 2013; Ven et al., 2019). Compared to traditional methods, the isotope tracer method permits us to consider all these belowground C allocation pathways. As rhizosphere respiration and rhizodeposition may account for a large proportion of the total C allocated belowground (Pausch and Kuzyakov, 2018), accounting for all belowground C allocation pathways will be required to accurately estimate CENA.

Belowground C allocation and CENA can vary greatly among plant species due to differences in root architecture and morphological traits, root exudates, mycorrhizal association, and the capacity of biological N_2 fixation (de Neergaard and Gorissen, 2004; Schmitt et al., 2013; Keller and Phillips, 2019). For example, legumes allocated more C to rhizosphere respiration compared to grasses, because of the extra energy and C demand for biological N_2 fixation by symbiotic rhizobia (Warembourg et al., 2003), while grasses may spend more C on dense fine roots or high rates of rhizodeposition for enhancing nutrient mobilization and uptake from the soil (Schmitt et al., 2013). The interspecific difference in belowground C allocation patterns will trigger different responses in nutrient acquisition, thereby influencing CENA between legumes and grasses.

Soil nutrient availability may also be an important factor influencing belowground C allocation and CENA. Using economic principles, it can be expected that nutrients become more C expensive for plants when their availability is low (Bloom et al., 1985). Most plants are limited by N, phosphorus (P), or both (Harpole et al., 2011; Du et al., 2020), and the amount of C that plants allocate belowground may strongly depend on which nutrient is limiting their growth. Although plant demand for P is lower than for N, belowground C allocation for P uptake may be higher than for N given that soil P availability is usually

much lower and less mobile compared to N (Vitousek et al., 2010). Plants secrete carboxylates to liberate inorganic P from mineral surfaces, or produce phosphatase extracellular enzymes to increase P mobilization through hydrolysis, when P availability to plants is limited (Lambers et al., 2008; Richardson et al., 2011; Wen et al., 2019). Furthermore, plants may increase belowground C allocation to support arbuscular mycorrhizal fungi to enhance P uptake under low P conditions (Smith et al., 2011; van der Heijden et al., 2015; Ven et al., 2019). Therefore, plants may allocate more belowground C to root exudates or rhizosphere respiration when plants are limited by P. Due to their capacity to fix N_2 from the atmosphere, the growth of legumes is more likely to be P-limited (Png et al., 2017), and belowground C allocation and CENA in legumes may therefore be more sensitive to P availability in soil compared to grasses.

Here, we assessed belowground C allocation and C efficiency for N and P acquisition in ryegrass (*Lolium perenne* L., C_3 grass) and clover (*Trifolium repens* L., legume) with and without P fertilization based on the same greenhouse experiment in Lu et al. (2020). By continuously labeling plants with CO_2 depleted in ^{13}C , we were able to quantify different components of belowground C allocation (root biomass, rhizosphere respiration, and rhizodeposition). We further used a ^{15}N natural abundance method to estimate biological N_2 fixation in clover and finally assessed CENA by comparing belowground C allocation to nutrient content in plant biomass after 58 days of growth. The objectives of this study were to (1) compare the difference in C efficiency for N (CENA_N) and for P (CENA_P) between ryegrass and clover and (2) assess how P fertilization affects CENA_N and CENA_P in ryegrass and clover. We hypothesized that (1) clover would have a higher C efficiency for N (CENA_N) compared to ryegrass because plant N acquisition through biological N_2 fixation is usually more C efficient compared to uptake from the soil (Fisher et al., 2010); (2) P fertilization would increase CENA_N in clover because P fertilization would increase biological N_2 fixation and reduce belowground C allocation associated with P uptake from the soil. However, P fertilization would increase N limitation in ryegrass, lowering CENA_N ; and (3) C efficiency for P (CENA_P) would be lower in clover because biological N_2 fixation would cause it to be more limited by P compared to ryegrass; for the same reason, P fertilization would increase CENA_P more in clover than in ryegrass.

MATERIALS AND METHODS

Experimental Design

Top soil (0–15 cm depth) was collected from a grassland at John Bruce Pye Farm in Camden, NSW, Australia ($33^\circ 56' 42''$ S, $150^\circ 40' 30''$ E). The soil is a red-brown chromosol (Isbell, 2002) (or Alfisol based on USDA Soil Taxonomy), with a pH of 5.4, 34% sand, 31% silt, and 35% clay. The $\delta^{13}\text{C}$ of soil organic C was -23.06‰ , and the organic C, total N, and total P concentrations were 28.8, 2.5, and 0.15 mg g^{-1} , respectively. The concentrations of soil mineral N (2 M KCl extraction) and available P ($0.03 \text{ M NH}_4\text{F}$ and 0.025 M HCl) were 58.0

and 8.7 mg kg⁻¹, respectively. Mesocosms consisted of bottom-capped polyvinyl chloride (PVC) pots (diameter 15 cm, height 20 cm) and sieved (4 mm) grassland soil (equivalent to 3.20 kg of oven-dried soil). After adjusting soil moisture content to 70% water-holding capacity (21% gravimetric soil moisture content), a modified Hoagland solution with macro- and micro-nutrients was added to all mesocosms [(NH₄)₂SO₄ 23.8, KNO₃ 25.7, Ca(NO₃)₂·4H₂O 11.9, MgCl₂·6H₂O 16.4, H₃BO₃ 0.08, ZnSO₄·7H₂O 0.2, CuSO₄·5H₂O 0.02, FeSO₄·7H₂O 0.25, and MnCl₂·4H₂O 0.3 g m⁻²]. The P was applied to the treatment with P as a KH₂PO₄ and K₂HPO₄ solution with an adjusted ratio to obtain a similar pH to the soil (4 g of P m⁻²), while for the treatment without P, a KCl solution was applied to eliminate the introduced K fertilization effect. These mesocosms (with and without P) were planted with either ryegrass (*Lolium perenne* L.) or clover (*Trifolium repens* L.) or were left unplanted (control). These two plant species are widely used to improve pastures in many temperate regions of the world. Besides the capacity of fixing N₂ from the atmosphere, ryegrass and clover also differ greatly in root morphological and architectural traits (Table 1), mycorrhizal infection (Zhu et al., 2000), and quantity and quality of root exudates (Lu et al., 2020), thereby showing different strategies of carbon allocation and nutrient acquisition. Six treatments were replicated four times. After germinating, each planted mesocosm was thinned to 20 plants.

This experiment lasted for 58 days in a controlled environmental facility at the Centre for Carbon, Water, and Food, University of Sydney, Camden (NSW). During the experimental period, the air temperature was kept at 25°C during the day and at 15°C during the night. The relative air humidity was kept at 60%, and artificial lights (Heliospectra, LX602C, 600 W, ~1 mmol m⁻² s⁻¹) went on for 12 h every day. The concentration of CO₂ was set at 800 ppm by injecting ¹³C-depleted CO₂ into the chamber. This was needed to reduce the δ¹³C value of CO₂ to a desired level and also to promote plants to grow faster, so that the labeled ¹³C signature could be detected in different pools or fluxes of belowground C allocation during a short cultivation period. Although a high level of CO₂ concentration would affect plant photosynthesis and thereby nutrient uptake dynamics, we are not aware that it would cause significant bias when comparing the treatment effects (species and P) under the same CO₂ concentration.

Moreover, our study provides novel insights into a mechanistic understanding of plant–soil interactions and is relevant given that global atmospheric CO₂ concentrations may reach 800 ppm or higher by the end of this century (Collins et al., 2013). We further note that we have used this method successfully in the past (e.g., Dijkstra and Cheng, 2007a; Canarini and Dijkstra, 2015). The δ¹³C value of CO₂ was maintained at $-20 \pm 0.3\%$ (mean ± standard deviation) throughout the experiment (measured on a G2131-i Analyzer, Picarro, Santa Clara, CA, United States). More details about continuous ¹³C-labeling method were reported in Lu et al. (2020). All mesocosms were randomly placed in the chamber and watered every 2 days to maintain soil moisture content at 70% water-holding capacity. The distribution of these mesocosms was randomly rotated every week to eliminate potential effects caused by spatial differences in light levels within the chamber.

Measurements

Total soil respiration was measured using a gas chamber method on 30, 44, and 58 days after planting (see full details in Lu et al., 2020). Briefly, at each gas sampling time, shoots were clipped at 1 cm above the soil surface and a non-transparent PVC chamber was sealed to each mesocosm. After removing initial CO₂ inside the mesocosm and chamber by circulating air inside the mesocosm through a soda lime column, a 12 mL gas sample was taken from the septum of the chamber at 0 h (T0), 1 h (T1), and 2 h (T2), respectively. These gas samples (T0, T1, and T2) were measured for CO₂ concentration and δ¹³C on a Delta V advantage isotope ratio mass spectrometer (IRMS) coupled to a Gasbench (Thermo Fisher Scientific, Bremen, Germany). As plants were continuously labeled with depleted ¹³C-CO₂ (δ¹³C = -20%), we were able to separate root-derived CO₂ (root respiration and microbial respiration of rhizodeposits) from soil-derived CO₂. At the end of the experiment, root samples were carefully picked from the mesocosm and soil samples were homogenized. The clipped shoots at each sampling time, hand-picked roots, and homogenized soil were measured for C%, N%, δ¹³C, and δ¹⁵N on a Delta V advantage IRMS coupled to a ConFlo IV and Flash HT (Thermo Fisher Scientific, Bremen, Germany). The plant samples were also measured for P concentration on the UV–VIS spectrophotometer (UVmini-1240), following the protocol described by Jackson (1958).

TABLE 1 | Mean diameter (MD), specific root length (SRL), specific root surface area (SRA), root tissue density (RTD), and root length density (RLD) of ryegrass and clover with and without P addition.

Treatment	MD, mm	SRL, m g ⁻¹	SRA, cm ² g ⁻¹	RTD, g cm ⁻³	RLD, m cm ⁻³
Ryegrass – P	0.23 ± 0.01b	220 ± 24a	1561 ± 119a	0.11 ± 0.01b	0.32 ± 0.04a
Ryegrass + P	0.24 ± 0.01b	208 ± 11a	1533 ± 58a	0.11 ± 0.01b	0.31 ± 0.03a
Clover – P	0.29 ± 0.01a	87 ± 7b	790 ± 42b	0.17 ± 0.01a	0.11 ± 0.01b
Clover + P	0.29 ± 0.01a	87 ± 4b	801 ± 31b	0.17 ± 0.01a	0.10 ± 0.01b
ANOVA (p-values)					
Species	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
P	0.601	0.676	0.906	0.61	0.76
Species × P	0.684	0.649	0.792	0.956	0.903

Values are shown as mean ± SE (n = 4). Two-way ANOVA p-values are shown.

Calculations

Belowground C allocation includes C for root growth (root biomass C), rhizosphere respiration (root-derived CO₂), and rhizodeposition (root-derived SOC). For clover, rhizosphere respiration also includes a C cost for biological N₂ fixation. We calculated rhizosphere respiration at each sampling date using a mass balance method based on δ¹³C signatures of CO₂ in planted and unplanted mesocosms (Lu et al., 2020) as follows:

$$C_{\text{root}} = C_{\text{total}} \times (\delta^{13}C_{\text{total}} - \delta^{13}C_{\text{soil}}) / (\delta^{13}C_{\text{root}} - \delta^{13}C_{\text{soil}}) \quad (1)$$

$$C_{\text{soil}} = C_{\text{total}} - C_{\text{root}} \quad (2)$$

where C_{total}, C_{soil}, and C_{root} are total belowground CO₂, soil-derived CO₂, and root-derived CO₂ in planted mesocosms, respectively. δ¹³C_{total} is the measured δ¹³C value of total belowground CO₂ in planted treatments. δ¹³C_{soil} is the mean δ¹³C value of soil respiration in the unplanted control. δ¹³C_{root} is the δ¹³C value of root-derived CO₂ in planted treatments, which was calculated based on the δ¹³C value of root tissue corrected by a fractionation factor of root-derived CO₂ relative to root tissue (−1.74‰ for grass and −2.67‰ for legume; Werth and Kuzyakov, 2010). We calculated the rhizosphere priming effect as the difference in soil-derived CO₂ between planted and unplanted control treatments (Lu et al., 2020).

Rhizosphere respiration during the whole 58-day experiment was then calculated using a linear extrapolation method based on the root-derived CO₂ at three sampling dates (30, 44, and 58 days after planting). It is noted that clipping before trapping belowground CO₂ may cause a decrease in root-derived CO₂ (Shahzad et al., 2012). Root-derived CO₂ was only measured during day time in this study. Root-derived CO₂ at night in wheat was sometimes lower than during the day, but at other times, night-time root-derived CO₂ was similar to day-time rhizosphere respiration (Kuzyakov and Cheng, 2001). Furthermore, we noted that the respiration of crowns and a negligible amount of shoot biomass were included in our measured rhizosphere respiration. Therefore, the calculated cumulative rhizosphere respiration during the entire experiment may have been somewhat overestimated, but unfortunately, we were unable to quantify this.

We calculated new root-derived SOC formed during the experiment (C_{new}) based on δ¹³C signatures of soil organic C at the start and end of the experiment in planted mesocosms (Dijkstra and Cheng, 2007b) as follows:

$$C_{\text{new}} = C_{\text{end}} \times (\delta^{13}C_{\text{initial}} - \delta^{13}C_{\text{end}}) / (\delta^{13}C_{\text{initial}} - \delta^{13}C_{\text{root}}) \quad (3)$$

where C_{initial} and C_{end} are the total amount of soil organic C at the beginning and end of the experiment, respectively. δ¹³C_{initial} is the δ¹³C value of C_{initial}, δ¹³C_{end} is the δ¹³C value of C_{end}, and δ¹³C_{root} is the δ¹³C value of root biomass.

Carbon efficiency for nutrient acquisition was calculated as plant nutrient content divided by belowground C allocation. Plant N and P contents were calculated by multiplying tissue biomass with tissue N and P concentration. Considering the C

cost for biological N₂ fixation in clover, we calculated CENA associated with belowground C allocation for plant nutrient uptake from the soil only, by subtracting C used for biological N₂ fixation from the total belowground C allocation (both for CENA_N and CENA_P) and subtracting biologically fixed N from the total plant N content (for CENA_N) in clover treatments as follows:

$$CENA_N = (N_{\text{plant}} - N_{\text{fix}}) / (C_{\text{below allocation}} - C_{\text{biological N fixation}}) \quad (4)$$

$$CENA_P = P_{\text{plant}} / (C_{\text{below allocation}} - C_{\text{biological N fixation}}) \quad (5)$$

where N_{plant} and P_{plant} are the N and P contents in the total plant biomass (shoots and roots) after day 58 plus the N and P contents in shoot biomass clipped on day 30 and 44, respectively. N_{fix} is the biologically fixed N in clover, C_{below allocation} is the total belowground C allocation, and C_{biological N fixation} is the C used for biological N₂ fixation. Carbon costs associated with biological N₂ fixation in legumes are relatively constant, ranging between 8 and 12 g C g^{−1} fixed N, depending on soil temperature (Fisher et al., 2010). Here, we used a value of 8 g C g^{−1} fixed N as the C cost for biological N₂ fixation in clover at 20°C. Biologically fixed N in clover was calculated using the ¹⁵N natural abundance method (Mia et al., 2018) as follows:

$$N_{\text{fix}} = N_{\text{clover}} \times (\delta^{15}N_{\text{ryegrass}} - \delta^{15}N_{\text{clover}}) / (\delta^{15}N_{\text{ryegrass}} - \delta^{15}N_{\text{bnf}}) \quad (6)$$

where N_{clover} is the N content in clover tissues, δ¹⁵N_{ryegrass} and δ¹⁵N_{clover} are the δ¹⁵N values of ryegrass (used as a reference plant) and clover tissues, respectively, and δ¹⁵N_{bnf} is the δ¹⁵N value of N-fixing plants completely relying on biological N₂ fixation (without N uptake from soil), which was estimated as −1.527‰ for clover (Mia et al., 2018).

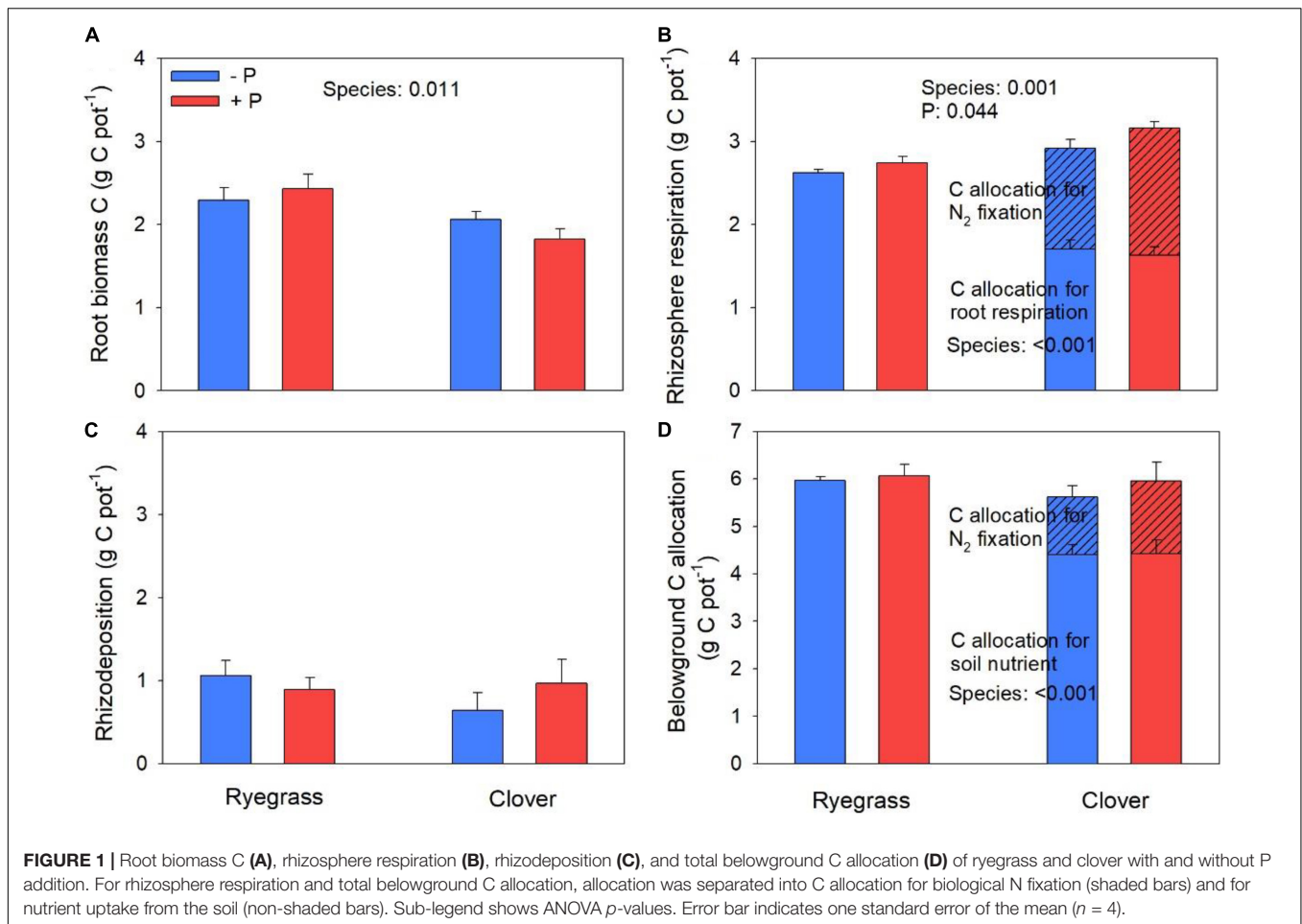
Statistical Analyses

Two-way ANOVA was applied to test the main and interactive effects of plant species and P fertilization on root biomass C, rhizosphere respiration, rhizodeposition, belowground C allocation, plant tissue δ¹⁵N, biologically fixed N, and CENA. The *post hoc* Tukey's honest significant difference (HSD) test was used to compare variables among ryegrass, ryegrass with P fertilization, clover, and clover with P fertilization treatments. Differences at *p* < 0.05 were considered significant, while differences between *p* > 0.05 and *p* < 0.1 were considered marginally significant. All statistical analyses were performed using the SPSS 20.0 (IBM SPSS Statistics 20, Armonk, United States).

RESULTS

Belowground C Allocation

Ryegrass showed larger root biomass C than clover (Figure 1A); clover showed larger rhizosphere respiration than ryegrass (Figure 1B); and the two species did not differ in rhizodeposition (Figure 1C). Due to the contrasting patterns of root biomass and rhizosphere respiration, there was no significant difference in belowground C allocation between the two species (Figure 1D).



Both ryegrass and clover allocated more belowground C to rhizosphere respiration (45 and 53%) and less to root biomass (40 and 34%) and rhizodeposition (15 and 13%) (Figure 1). For clover, the C cost for biological N₂ fixation accounted on an average for 45% of rhizosphere respiration and 24% of total belowground C allocation, respectively. When excluding C cost for biological N₂ fixation, clover showed lower rhizosphere respiration (Figure 1B) and less belowground C allocation than ryegrass (Figure 1D). P fertilization increased rhizosphere respiration (on an average by 6%; Figure 1B) but did not significantly influence the total belowground C allocation of both species (Figure 1D).

Plant Nutrient Acquisition and Carbon Efficiency for Nutrient Acquisition

Plant N and P contents were reported in Lu et al. (2020). Briefly, clover had higher plant N and P contents than ryegrass (104 and 53% higher, respectively). On average, 37% of plant N in clover was biologically fixed by rhizobia from the atmosphere (Table 2). After subtracting biologically fixed N, non-fixed N in clover was still higher than in ryegrass (28%). Phosphorus fertilization significantly increased plant P content in both species (10%), marginally increased biologically fixed N in clover (27%,

Table 2) and significantly decreased non-fixed N in both species (11%). CEN_{N} (including C cost for biological N₂ fixation) was higher for clover than for ryegrass (Figure 2A). When excluding C cost for biological N₂ fixation, clover still showed a higher CEN_{N} than ryegrass (Figure 2B), while CEN_{P} was also higher for clover (Figure 2C), indicating that clover obtained more N and P from soil with less belowground C. Phosphorus addition increased CEN_{P} (Figure 2C) but decreased CEN_{N} in both species (Figure 2B), although the effects were marginally significant.

DISCUSSION

Half of the total belowground C allocation was allocated to rhizosphere respiration (including root respiration and rhizosphere microbial respiration of rhizodeposits), suggesting that autotrophic respiration plays an important role in plant belowground C allocation and the total soil CO₂ efflux (Hopkins et al., 2013). Rhizodeposition remaining in soil accounted for the lowest fraction of belowground C allocation (an average of 14%), but actual rhizodeposition rates must be higher considering that most of the rhizodeposition is lost as CO₂ via rapid microbial decomposition (Pausch et al., 2013;

TABLE 2 | Plant $\delta^{15}\text{N}$ values in shoot and root biomass of ryegrass and clover, and biologically fixed N in clover with and without P fertilization (T1, Day 30; T2, Day 44; T3, Day 58).

Treatments	Plant $\delta^{15}\text{N}$				Fixed N (mg pot ⁻¹)
	T1-shoot $\delta^{15}\text{N}$	T2-shoot $\delta^{15}\text{N}$	T3-shoot $\delta^{15}\text{N}$	Root $\delta^{15}\text{N}$	
Ryegrass – P	2.57 ± 0.06ab	2.35 ± 0.12a	1.59 ± 0.07a	1.63 ± 0.22a	0
Ryegrass + P	2.28 ± 0.08b	1.55 ± 0.13bc	1.06 ± 0.12b	1.31 ± 0.04ab	0
Clover – P	3.08 ± 0.31a	1.97 ± 0.14ab	–0.43 ± 0.06c	1.02 ± 0.17ab	151 ± 12.2
Clover + P	2.69 ± 0.1ab	1.07 ± 0.15c	–1.01 ± 0.11d	0.77 ± 0.13b	191 ± 16.4
ANOVA (p-values)					
Species	0.016	0.007	< 0.001	0.003	–
P	0.065	< 0.001	< 0.001	0.088	–
Species × P	0.770	0.712	0.771	0.836	–

Values are shown as mean ± SE (n = 4). Two-way ANOVA p-values are shown.

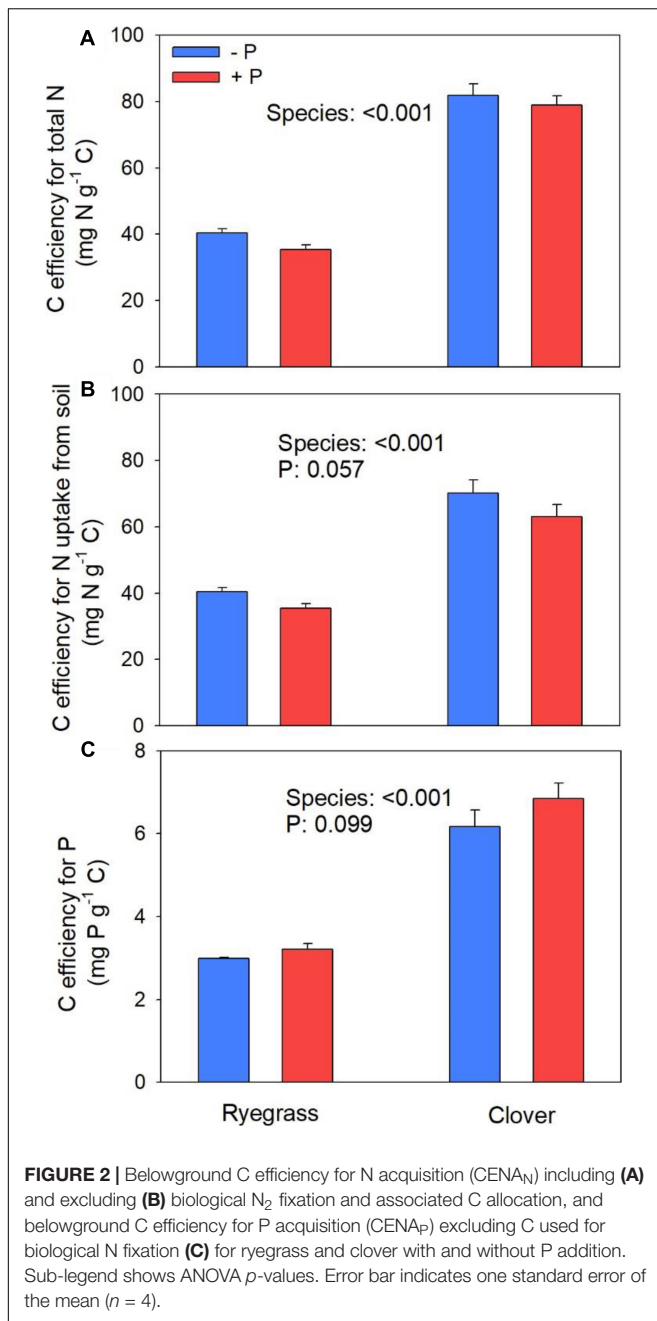
Pausch and Kuzyakov, 2018; Liu et al., 2019), which in our case was included in rhizosphere respiration. Root biomass accounted for 37% of total belowground C allocation, which was smaller than rhizosphere respiration and rhizodeposition. These results suggest that the assessment of belowground C allocation based only on root biomass is somewhat biased and that rhizosphere respiration and rhizodeposition cannot be ignored. We acknowledge that our estimates of rhizosphere respiration were based on only three flux measurements and that they were done during the day only, and as such have the greatest uncertainty that needs further investigation. Nevertheless, the distribution of belowground C among different pools or fluxes is to some degree consistent with the C allocation patterns for grassland species synthesized by Pausch and Kuzyakov (2018).

Although clover allocated less C to root biomass, it showed greater rhizosphere respiration than ryegrass, possibly because biological N₂ fixation by clover requires C. Indeed, when accounting for C allocation toward biological N₂ fixation and assuming that this C would eventually be respired as CO₂, there was a larger rhizosphere respiration in ryegrass than in clover instead (Figure 1B). Thus, C allocation toward biological N₂ fixation was an important component of rhizosphere respiration for clover. Previous studies also suggested that legumes had a higher demand for assimilated C as indicated by higher rhizosphere respiration than grasses (Warembourg et al., 2003; Schmitt et al., 2013). Furthermore, we estimated that the C cost for biological N₂ fixation was about 8 g C g⁻¹ N (C cost at 20°C) according to Fisher et al. (2010), while the C allocation for plant N uptake from soil in clover was then 14 g C g⁻¹ N (inverse of CENA_N, Figure 2B). This result suggests that biological N₂ fixation by the legume is a relatively C efficient way to acquire N as compared to plant N uptake from soil. Even if the C cost for biological N₂ fixation is higher than what we assumed (e.g., assuming 10 or 12 g C g⁻¹ fixed N, respectively; Fisher et al., 2010), the C cost for biological N₂ fixation would still be cheaper than or similar to that for N uptake from soil in clover (e.g., C allocation for plant N uptake would then be 13.3 or 12.3 g C g⁻¹ N, respectively). Clearly, more work is needed to compare the C cost for biological N₂ fixation vs. N uptake. Nevertheless, our results may explain why N₂-fixing plants often compete

with non-fixing plants, particularly under the condition of low N availability (Vitousek and Howarth, 1991; Crews, 1999; Menge et al., 2017; Wang et al., 2022).

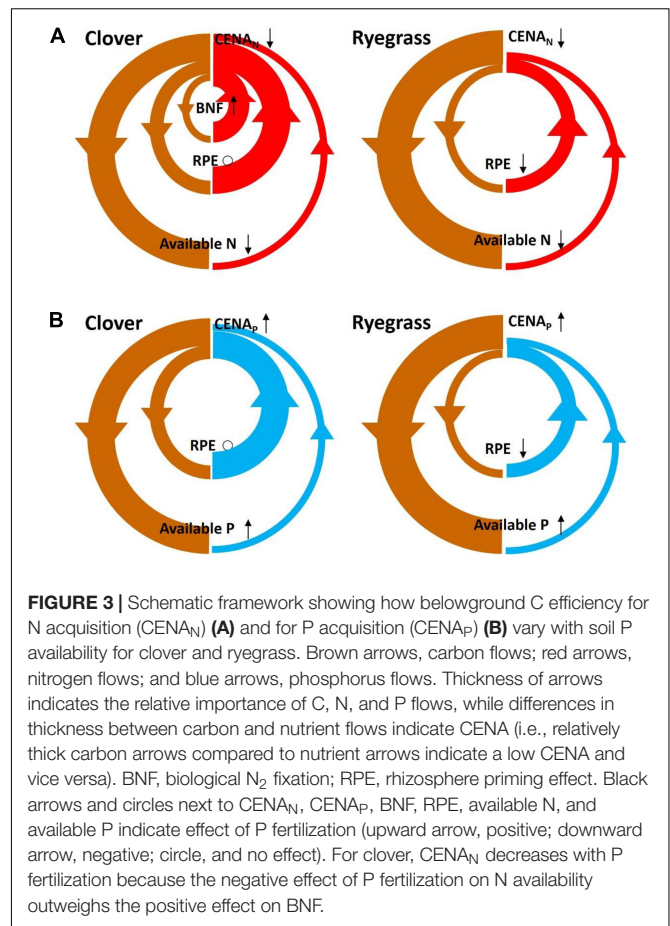
Consistent with our hypothesis, clover had a higher CENA_N than ryegrass because, as discussed above, less C was required for biological N₂ fixation than for N uptake from soil. However, CENA_N of clover was still higher than ryegrass after we accounted for the C cost associated with biological N₂ fixation. In contrast to our hypothesis, CENA_P was also higher for clover than for ryegrass. Possibly, the greater rhizosphere priming effect on soil organic matter decomposition that we observed for clover by the end of the experiment in another study may contribute to the higher CENA_N and CENA_P (Lu et al., 2020). By the end of the experiment, available forms of N in soil were extremely low (less than 7 mg N pot⁻¹ or 2 mg N kg⁻¹ soil), and likely very C expensive to take up by both ryegrass and clover. Therefore, stimulation of soil organic matter decomposition by root exudates and subsequent release of N (and P) for plant uptake may be a very C-efficient way for plants to acquire nutrients from the soil (Figure 3; Wang et al., 2022). Previous studies also suggested that legume species could produce larger rhizosphere priming effects than non-legume species (Cheng et al., 2003; Drake et al., 2013). Alternatively, the higher CENA_P in clover than in ryegrass may also be attributed to the tendency of legumes to cause greater acidification in the rhizosphere and exude carboxylates to mobilize and increase concentrations of inorganic P through dissolution or desorption (Hinsinger, 2001; Nuruzzaman et al., 2006).

In contrast to our hypothesis, P fertilization slightly decreased the CENA_N in ryegrass and clover, suggesting that P fertilization caused these plants to allocate more belowground C to acquire N from soil. In this study, although biological N₂ fixation in clover marginally increased with P fertilization (Table 2), the increase of this relatively cheap form of N acquisition was apparently not enough to counter the increase in belowground C allocation for soil N uptake. Possibly, P fertilization may have exacerbated soil N limitation by increasing microbial N immobilization (Blanes et al., 2012; Mehnaz et al., 2019), and thus did not improve belowground C allocation. Indeed, microbial biomass N measured at the end of the experiment was significantly higher



with P fertilization (an average of 17%; Lu et al., 2020). In turn, increased microbial N immobilization with P fertilization may thus reduce soil available N, thereby making it more C expensive for plant uptake (Figure 3). It has also been suggested that C allocation could increase to maintain plant N uptake at low N conditions (Brzostek et al., 2014; Perkowski et al., 2021). Our results further imply that plant C allocation belowground for N uptake depends on P availability.

Phosphorous fertilization marginally increased the CEN_{P} for both species, indicating that the P uptake may become somewhat less C expensive with increased soil P availability (Figure 3). This result is consistent with the resource optimization hypothesis that



plants allocate more C to acquire limited resources (McMurtrie and Dewar, 2013). Previous studies also found that belowground C investment in P acquisition was more efficient with P fertilization (Ven et al., 2019). We expected that CEN_{P} would increase more for clover than for ryegrass given that growth of the N-fixing clover would be more limited by P than ryegrass (Png et al., 2017). However, we found no support for this (no significant species \times P interaction), but we should note that the increases in CEN_{P} with P fertilization were relatively small and only marginally significant. Clearly, more work is needed for an in-depth understanding of the mechanisms underlying the CEN_{N} and CEN_{P} of grasses and legumes under different N and P availabilities.

While we did not investigate interspecific interactions on CEN_{N} , the CEN_{N} concept may have significant implications for plant community dynamics and competition for nutrients in legume-grass mixtures (Raven et al., 2018; Wang et al., 2022). When legumes have a higher CEN_{N} compared to grasses, they may temporarily outcompete grasses for soil nutrients until conditions arise that reduce CEN_{N} for legumes more than for grasses (e.g., under drought or high levels of soil N). Changes in CEN_{N} with time would also depend on how flexible plants are in switching C allocation toward different nutrient acquisition strategies (Fisher et al., 2010). Furthermore, interspecific competition for nutrients by itself, as well as the

transfer of biologically fixed N from legume to grass, could potentially influence the CENA of individual species in grassland mixtures, which could help explain the often observed temporal dynamics in legume and grass abundance in mixed pastures (Ledgard and Steele, 1992). Further research is therefore needed to investigate the role of CENA in plant community dynamics in legume-grass mixtures.

CONCLUSION

In our study, we quantified the belowground C allocation and its efficiency for N and P acquisition ($CENA_N$ and $CENA_P$, respectively). We showed that clover had higher $CENA_N$ and $CENA_P$ than ryegrass, even after accounting for the relatively low C costs associated with biological N_2 fixation, possibly because of the distinct rhizosphere priming on soil organic matter decomposition. Furthermore, P fertilization decreased $CENA_N$, possibly *via* exacerbating soil N limitation, while P fertilization increased $CENA_P$ because plant P acquisition was more efficient with increased P availability. Current modeling studies have indicated that net primary productivity and soil C storage are strongly associated with variation in the belowground C allocation for nutrient uptake (Fisher et al., 2010; Brzostek et al., 2014; Shi et al., 2016). Yet, estimates of $CENA_N$ and $CENA_P$ are lacking. To the best of our knowledge, this is one of the first studies to provide estimates for these parameters. We acknowledge that our study is based on two plant species only and in one soil type only, and that variation in CENA should be examined across a larger set of plant species, communities, and soil types during a relatively long period. Nevertheless, we believe that better understanding of $CENA_N$ and $CENA_P$ will

help not only improve global C cycling model predictions but also identify management practices to increase yield and fertilizer use efficiency in agricultural systems.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JL and FD designed the experiment. JL, JY, and CK performed the experiment. JL analyzed the data. JL, LY, PW, WC, and FD wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Australian Research Council (DP190102262), the National Natural Science Foundation of China (32001386), and a visiting scholarship to JL from the Joint Ph.D. Training Program by the University of Chinese Academy of Sciences.

ACKNOWLEDGMENTS

We thank Milad Bagheri Shirvan for laboratory assistance.

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