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

Jiayu Lu¹², Jinfeng Yang³, Claudia Keitel², Liming Yin¹, Peng Wang¹*, Weixin Cheng⁴ and Feike A. Dijkstra*¹

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

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 belowground C allocation 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 (Warenbourg 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 ($33^\circ56'42''$ S, $150^\circ40'30''$ 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 $\delta^{13}$C of soil organic C was $-23.06\%_o$, 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 M NH$_4$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 \([\text{NH}_4]_2\text{SO}_4\; 23.8, \text{KNO}_3\; 25.7, \text{Ca(NO}_3\)_2\cdot 4\text{H}_2\text{O}\; 11.9, \text{MgCl}_2\cdot 6\text{H}_2\text{O}\; 16.4, \text{H}_3\text{BO}_3\; 0.08, \text{ZnSO}_4\cdot 7\text{H}_2\text{O}\; 0.2, \text{CuSO}_4\cdot 5\text{H}_2\text{O}\; 0.02, \text{FeSO}_4\cdot 7\text{H}_2\text{O}\; 0.25, \text{MnCl}_2\cdot 4\text{H}_2\text{O}\; 0.3\, \text{g m}^{-2}\)]. The P was applied to the treatment with P as a KH\(_2\)PO\(_4\) and K\(_2\)HPO\(_4\) 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 for the treatment without P, a KCl solution was applied 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 CO\(_2\) 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 CO\(_2\) concentration and \(\delta^{13}\)C 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 \(^{13}\)C-CO\(_2\) (\(\delta^{13}\)C = \(-20\%e\) \(\pm 3\%e\)), we were able to separate root-derived CO\(_2\) (root respiration and microbial respiration of rhizodeposits) from soil-derived CO\(_2\). 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%, \(\delta^{13}\)C, and \(\delta^{15}\)N 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 x 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 CO$_2$), and rhizodeposition (root-derived SOC). For clover, rhizosphere respiration also includes a C cost for biological N$_2$ fixation. We calculated rhizosphere respiration at each sampling date using a mass balance method based on $\delta^{13}$C signatures of CO$_2$ in planted and unplanted mesocosms (Lu et al., 2020) as follows:

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

(1)

$$C_{\text{soil}} = C_{\text{total}} - C_{\text{root}}$$

(2)

where $C_{\text{total}}$, $C_{\text{soil}}$, and $C_{\text{root}}$ are total belowground CO$_2$, soil-derived CO$_2$, and root-derived CO$_2$ in planted mesocosms, respectively. $\delta^{13}C_{\text{total}}$ is the measured $\delta^{13}$C value of total belowground CO$_2$ in planted treatments. $\delta^{13}C_{\text{soil}}$ is the mean $\delta^{13}$C value of soil respiration in the unplanted control. $\delta^{13}C_{\text{root}}$ 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; Werth and Kuzyakov, 2010). We calculated the rhizosphere priming effect as the difference in soil-derived CO$_2$ 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 CO$_2$ at three sampling dates (30, 44, and 58 days after planting). It is noted that clipping before trapping belowground CO$_2$ may cause a decrease in root-derived CO$_2$ (Shahzad et al., 2012). Root-derived CO$_2$ was only measured during day time in this study. Root-derived CO$_2$ at night in wheat was sometimes longer than during the day, but at other times, night-time root-derived CO$_2$ 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 underestimated, but unfortunately, we were unable to quantify this.

We calculated new root-derived SOC formed during the experiment ($C_{\text{new}}$) based on $\delta^{13}$C signatures of soil organic C at the start and end of the experiment in planted mesocosms (Dijkstra and Cheng, 2007b) as follows:

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

(3)

where $C_{\text{initial}}$ and $C_{\text{end}}$ are the total amount of soil organic C at the beginning and end of the experiment, respectively. $\delta^{13}C_{\text{initial}}$ is the $\delta^{13}$C value of $C_{\text{initial}}$, $\delta^{13}C_{\text{end}}$ is the $\delta^{13}$C value of $C_{\text{end}}$, and $\delta^{13}C_{\text{root}}$ is the $\delta^{13}$C 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 N$_2$ 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 N$_2$ 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:

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

(4)

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

(5)

where $N_{\text{plant}}$ and $P_{\text{plant}}$ 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 clamped on day 30 and 44, respectively. $N_{\text{fix}}$ is the biologically fixed N in clover, $C_{\text{below allocation}}$ is the total belowground C allocation, and $C_{\text{biological N fixation}}$ is the C used for biological N$_2$ fixation. Carbon costs associated with biological N$_2$ 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 N$_2$ fixation in clover at 20°C. Biologically fixed N in clover was calculated using the $\delta^{15}$N natural abundance method (Mia et al., 2018) as follows:

$$N_{\text{fix}} = N_{\text{clover}} - (\delta^{15}N_{\text{ryegrass}} - \delta^{15}N_{\text{clover}})/(\delta^{15}N_{\text{ryegrass}} - \delta^{15}N_{\text{N2fix}})$$

(6)

where $N_{\text{clover}}$ is the N content in clover tissues, $\delta^{15}N_{\text{ryegrass}}$ and $\delta^{15}N_{\text{clover}}$ are the $\delta^{15}$N values of ryegrass (used as a reference plant) and clover tissues, respectively, and $\delta^{15}N_{\text{N2fix}}$ is the $\delta^{15}$N value of N-fixing plants completely relying on biological N$_2$ 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 $\delta^{15}$N, 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 N fixation accounted on an average for 45% of rhizosphere respiration and 24% of total belowground C allocation, respectively. When excluding C cost for biological N\textsubscript{2} 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).

**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 CO\textsubscript{2} 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 CO\textsubscript{2} via rapid microbial decomposition (Pausch et al., 2013; Table 2) and significantly decreased non-fixed N in both species (11%). CENA\textsubscript{N} (including C cost for biological N\textsubscript{2} fixation) was higher for clover than for ryegrass (Figure 2A). When excluding C cost for biological N\textsubscript{2} fixation, clover still showed a higher CENA\textsubscript{P} than ryegrass (Figure 2B), while CENA\textsubscript{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 CENA\textsubscript{P} (Figure 2C) but decreased CENA\textsubscript{N} in both species (Figure 2B), although the effects were marginally significant.
ANOVA (× P 0.065) our results may explain why N_{2} the C cost for biological N allocation for plant N uptake would then be 13.3 or 12.3 g C than or similar to that for N uptake from soil in clover (e.g., C_{N uptake from soil in clover was then 14 g C g^{-1} for biological N as compared to plant N uptake from soil. Even if the C cost fixation by the legume is a relatively C efficient way to acquire fixation was an important component of rhizosphere respiration (Warembourg et al., 2003; a higher demand for assimilated C as indicated by higher fixation was about 8 g C g^{-1} C) (Blanes et al., 2012; Mehnaz et al., 2019), and thus did not account for C allocation toward biological N_{2} (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, Figure 2B). This result suggests that biological N_{2} 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 N_{2} 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 N_{2} 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 N_{2} fixation vs. N uptake. Nevertheless, our results may explain why N_{2}-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 than ryegrass because, as discussed above, less C was required for biological N_{2} fixation than for N uptake from soil. However, CENA of clover was still higher than ryegrass after we accounted for the C cost associated with biological N_{2} 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 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 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 N_{2} 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

### Table 2 | Plant δ^{15}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 98).

| Treatments | Plant δ^{15}N | Fixed N (mg pot^{-1}) |
|------------|---------------|-----------------------|
|            | T1-shoot | T2-shoot | T3-shoot | Root |               |
| Ryegrass – P | 2.57 ± 0.06ab | 2.35 ± 0.12a | 1.59 ± 0.07a | 1.63 ± 0.22a | 0 |
| Ryegrass + P | 2.28 ± 0.06b | 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 ± 0.06bc | 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 | P | Species × P |
|------------|---------|---|-------------|
|            | 0.016   | 0.065 | 0.770       |
|            | 0.007   | < 0.001 | 0.712       |
|            | < 0.001 | 0.001 | 0.771       |
|            | 0.003   | 0.088 | 0.836       |
|            | –       | –       | –           |

Values are shown as mean ± SE (n = 4). Two-way ANOVA p-values are shown.
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 CENA_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 investment in P acquisition was more efficient with P fertilization (Ven et al., 2019). We expected that CENA_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 × P interaction), but we should note that the increases in CENA_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 CENA_N and CENA_P of grasses and legumes under different N and P availabilities.

While we did not investigate interspecific interactions on CENA, the CENA 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 CENA compared to grasses, they may temporarily outcompete grasses for soil nutrients until conditions arise that reduce CENA for legumes more than for grasses (e.g., under drought or high levels of soil N). Changes in CENA 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 N2 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.

REFERENCES

Blanes, M. C., Emmett, B. A., Viñegla, B., and Carreira, J. A. (2012). Alleviation of P limitation makes tree roots competitive for N against microbes in a N-saturated conifer forest: a test through P fertilization and 15N labelling. Soil Biol. Biochem. 48, 51–59. doi: 10.1016/j.soilbio.2012.01.012

Bloom, A. J., Chapin, F. S., and Mooney, H. A. (1985). Resource limitation in plants: An economic analogy. Annu. Rev. Ecol. Syst. 16, 363–392. doi: 10.1146/annurev.es.16.110185.002051

Brzostek, E. R., Fisher, J. R., and Phillips, R. P. (2014). Modeling the carbon cost of plant nitrogen acquisition: mycorrhizal trade-offs and multipath resistance uptake improve predictions of retranslocation. J. Geophys. Res. Biogeosci. 119, 1684–1697. doi: 10.1002/2014jg002660

Canarini, A., and Dijkstra, F. A. (2015). Dry-rewetting cycles regulate wheat carbon rhizodeposition, stabilization and nitrogen cycling. Soil Biol. Biochem. 81, 195–203. doi: 10.1016/j.soilbio.2014.11.014

Cheng, W., Johnson, D. W., and Fu, S. (2003). Rhizosphere effects on decomposition: control of plant species, phenology, and fertilization. Soil Sci. Soc. Am. J. 67, 1418–1427. doi: 10.2136/sssaj2003.1418

Collins, M., Knutti, R., Arblaster, J., Dufresne, J.-L., Fichefet, T., Friedlingstein, P., et al. (2013). “Long-term Climate Change: Projections, Commitments and Irreversibility;” in Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, eds T. F. Stocker, D. Qin, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, et al. (Cambridge: Cambridge University Press).

Crews, T. E. (1999). The presence of nitrogen fixing legumes in terrestrial communities: evolutionary vs ecological considerations. Biogeochemistry 46, 233–246. doi: 10.1007/BF01007581

de Neergaard, A., and Gorissen, A. (2004). Carbon allocation to roots, rhizodeposits and soil after pulse labelling: a comparison of white clover (Trifolium repens L.) and perennial ryegrass (Lolium perenne L.). Biol. Fertil. Soils 39, 228–234. doi: 10.1007/s00374-003-0699-x

Dijkstra, F. A., and Cheng, W. (2007a). Moisture modulates rhizosphere effects on C decomposition in two different soil types. Soil Biol. Biochem. 39, 2264–2274. doi: 10.1016/j.soilbio.2007.03.026

Dijkstra, F. A., and Cheng, W. (2007b). Interactions between soil and tree roots accelerate long-term soil carbon decomposition. Ecol. Lett. 10, 1046–1053. doi: 10.1111/j.1461-0248.2007.01995.x

Drake, J. E., Darby, B. A., Giasson, M. A., Kramer, M. A., Phillips, R. P., and Finzi, A. C. (2013). Stoichiometry constrains microbial response to root exudation-insights from a model and a field experiment in a temperate forest. Biogeosci. 10, 821–838. doi: 10.5194/bg-10-821-2013

Du, E., Terrer, C., Pellegrini, A. F. A., Ahlstrom, A., van Lissa, C. J., Zhao, X., et al. (2020). Global patterns of terrestrial nitrogen and phosphorus limitation. Nat. Geosci. 13, 221–226. doi: 10.1038/s41561-019-0530-4

Fisher, J. B., Sitch, S., Malm, Y., Fisher, R. A., Huntingford, C., and Tan, S. Y. (2010). Carbon cost of plant nitrogen acquisition: a mechanistic, globally applicable model of plant nitrogen uptake, retranslocation, and fixation. Glob. Biogeochem. Cycles 24:GB1014. doi: 10.1029/2009gb003621

Harpole, W. S., Ngai, J. T., Cleland, E. E., Seabloom, E. W., Borer, E. T., Bracken, M. E. S., et al. (2011). Nutrient co-limitation of primary producer communities. Ecol. Lett. 14, 852–862. doi: 10.1111/j.1461-0248.2011.01651.x

Hinsinger, P. (2001). Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. Plant Soil 237, 173–195.

Hopkins, F., Gonzalez-Meler, M. A., Flower, C. E., Lynch, D. J., Czimczik, C., Tang, J., et al. (2013). Ecosystem-level controls on root-rhizosphere respiration. N. Phytol. 199, 339–351. doi: 10.1111/nph.12271
Belowground Carbon Efficiency for Nutrients

Lu et al.

Isbell, R. F. (2002). The Australian Soil Classification. Collingwood, Vic, Australia: CSIRO Pub.

Jackson, M. L. (1958). Soil Chemical Analysis. Englewood Cliffs, NJ: Prentice-Hall, Inc.

Keller, A. B., Borrerstek, E. R., Craig, M. E., Fisher, J. B., and Phillips, R. P. (2021). Root-derived inputs are major contributors to soil carbon in temperate forests, but vary by mycorrhizal type. *Ecol. Lett.* 24, 626–635. doi: 10.1111/ele.14651

Keller, A. B., and Phillips, R. P. (2019). Relationship between belowground carbon allocation and nitrogen uptake in saplings varies per plant mycorrhizal type. *Front. Forests Glob. Chang.* 2:81. doi: 10.3389/ffr.2019.00081

Kuziyakov, Y., and Cheng, W. (2001). Photosynthesis controls of rhizosphere respiration and organic matter decomposition. *Soil Biol. Biochem.* 33, 1915–1925. doi: 10.1016/S0038-0717(01)00117-1

Lambers, H., Raven, J. A., Shaver, G. R., and Smith, S. E. (2008). Plant nutrient-Soil Chemical Analysis: The Australian Soil Classification. Collingwood, Vic, Australia: CSIRO Pub.

Isbell, R. F. (2002). *Lu et al. Belowground Carbon Efficiency for Nutrients*.

Ledgard, S. F., and Steele, K. W. (1992). Biological nitrogen fixation in mixed legume/grass pastures. *Soil Biol. Biochem.* 24, 626–635. doi: 10.1016/0038-0717(92)90095-2

Richardson, A. E., Lynch, J. P., Ryan, P. R., Delhaize, E., Smith, F. A., Smith, S. E., et al. (2011). Plant and microbial strategies to improve the phosphorus efficiency of agriculture. *Plant Soil* 349, 121–156. doi: 10.1007/s11104-011-0950-4

Schmitt, A., Pausch, J., and Kuziyakov, Y. (2013). C and N allocation in soil under ryegrass and alfalfa estimated by 13C and 15N labelling. *Plant Soil* 368, 581–590. doi: 10.1007/s11104-012-1536-5

Shahzad, T., Chenu, C., Repinczay, C., Mougin, C., Ollier, J., and Fontaine, S. (2012). Plant clipping decelerates the mineralization of recalcitrant soil organic matter under multiple grassland species. *Soil Biol. Biochem.* 51, 73–80. doi: 10.1016/j.soilbio.2012.04.014

Shi, M., Fisher, J. B., Borrerstek, E. R., and Phillips, R. P. (2016). Carbon cost of plant nitrogen acquisition: global carbon cycle impact from an improved plant nitrogen cycle in the Community Land Model. *Glob. Change Biol.* 22, 1299–1314. doi: 10.1111/gcb.13131

Smith, S. E., Jakobsen, I., Grönlund, M., and Smith, F. A. (2011). Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. *Plant Physiol.* 156, 1050–1057. doi: 10.1104/pp.111.175381

van der Heijden, M. G. A., Martin, F. M., Selosse, M. A., and Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *N. Phytol.* 205, 1406–1423. doi: 10.1111/nph.13288

Ven, A., Verlinden, M. S., Verbruggen, E., and Vicca, S. (2019). Experimental evidence that phosphorus fertilization and arbuscular mycorrhizal symbiosis can reduce the carbon cost of phosphorus uptake. *Funct. Ecol.* 33, 2215–2225. doi: 10.1111/1365-2435.13452

Vicca, S., Luysaert, S., Peñuelas, J., Campioli, M., Chapin, F. S., Ciais, P., et al. (2012). Fertile forests produce biomass more efficiently. *Ecol. Lett.* 15, 520–526. doi: 10.1111/j.1461-0248.2012.01775.x

Vitousek, P. M., and Howarth, R. W. (1991). Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* 13, 87–115. doi: 10.1016/0169-5303(91)90027-2

Wang, R., Lu, J., Jiang, Y., and Dijkstra, F. A. (2022). Carbon efficiency for nutrient acquisition (CENA) by plants: role of nutrient availability and microbial symbionts. *Plant Soil* 310:11104-022-05347-y

Warming, E. R., Roumet, C., and Lafont, F. (2003). Differences in rhizosphere carbon-partitioning among plant species of different families. *Plant Soil* 256, 347–357. doi: 10.1023/a:1026147622800

Wen, Z., Li, H., Shen, Q., Tang, X., Xiong, C., Li, H., et al. (2019). Tradeoffs among root morphology, exudation and mycorrhizal symbioses for phosphorus-acquisition strategies of 16 crop species. *N. Phytol.* 223, 882–895. doi: 10.1111/nph.15833

Wirth, M., and Kuziyakov, Y. (2010). 13C fractionation at the root- microorganisms-soil interface: a review and outlook for partitioning studies. *Soil Biol. Biochem.* 42, 1372–1384. doi: 10.1016/j.soilbio.2010.04.009

Zhu, B., Gutknecht, J. L. M., Herman, D. J., Keck, D. C., Firestone, M. K., and Cheng, W. (2014). Rhizosphere priming effects on soil carbon and nitrogen mineralization. *Soil Biol. Biochem.* 76, 183–192. doi: 10.1016/j.soilbio.2014.04.033

Zhu, Y. G., Lidlaw, A. S., Christie, P., and Hammond, M. E. R. (2000). The specificity of arbuscular mycorrhizal fungi in perennial ryegrass-white clover pasture. *Agric. Ecosystems Environ.* 77, 211–218. 87-80 doi: 10.1016/S0167-8809(99)

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher’s Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Lu, Yang, Keitel, Yin, Wang, Cheng and Dijkstra. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.