A novel methodology to track nitrogen transfer in a grass-legume mixture using enriched 15N2

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Abstract

Background and Aims – Legumes are a potentially important N source in pasture systems, but quantifying the transfer of biologically fixed N from the legume to the grass component is difficult. A greenhouse H-pot system was developed to directly estimate biological N_2 fixation (BNF) and belowground N transfer using $^{15}N_2$. The system was tested with annual ryegrass (*Lolium multiflorum* L.) and crimson clover (*Trifolium incarnatum* L.).

Methods – Legume and grass root systems growing in either individual or H pots were exposed to $^{15}N_2$. Control H pots were separated by mesh to prevent contact between roots and mycorrhizae from each side of the pot. To reduce volume demand and avoid cross-contamination in the greenhouse, the gas was supplied through underground tubes in the root zone.

Results – Ryegrass and clover were enriched in ^{15}N when the respective root system was supplied with $^{15}N_2$. Ryegrass was also enriched when clover roots were supplied with the gas and there was free root and mycorrhizal contact between both sides of the H pot, but not when this contact was precluded. Plants grown singly did not enrich when the gas was not supplied to their root systems.

Conclusions – The H-pot construction allows the evaluation of belowground transmission, an important mechanism of N transfer. The method of gradually supplying $^{15}N_2$ directly to the root system may be a valuable labeling technique for monitoring the transfer of nitrogen. The lack of enrichment when plants were not directly supplied indicates negligible atmospheric enrichment. Ryegrass enrichment, when supplied with the gas, suggests BNF by other mechanisms.

Introduction

Legumes enhance the provision of ecosystem services when included in mixtures with grasses in forage systems due to their association with rhizobia and consequent BNF (Sollenberger et al. 2019). The extent of BNF in grasslands is dependent on forage accumulation, the proportion of legume in the botanical composition, legume N concentration, and the proportion of N_2 that is derived from the atmosphere in the legume biomass (Boller and Nosberger 1987). In grass-legume swards, N transfer from legume to companion species can occur both through belowground and aboveground processes, with plant litter and animal excreta generally considered the main pathways for this transfer (Dubeux et al. 2007; Ledgard 1991; Rouquette and Smith 2010). Lesser-known transfer pathways include plant-associated arbuscular-mycorrhizal fungi; exudation and leakage from living nodules and roots; root cell and nodule sloughing in response to defoliation, and through root and nodule death and decay (Dhamala et al. 2017; Fustec et al. 2010; He et al. 2009; Paynel et al. 2008; Thilakarathna et al. 2016).

Tracking both N_2 fixation and N transfer from legume to grasses in mixed swards has been challenging, and several techniques have been suggested. The heavy isotope technique using ^{15}N has been used for the last 60 years for BNF research. The most common method for labeling is the addition of ^{15}N-

enriched fertilizer. The use of ^{15}N-enriched N_2 gas is less common due to its cost, but its use avoids some of well-known problems linked to using ^{15}N-enriched fertilizers (Russelle 2008). An advantage of gas is its use in an enclosed and sealed chamber assures that the fixed N is coming from the labelled $^{15}N_2$ gas via BNF (Unkovich et al. 2008).

Thus far, there have been no reported studies regarding the infusion of ^{15}N-enriched N_2 gas directly into the root zone of grasses and legumes growing in connected pots (i.e., H-shaped pots). The overall objective of this study was to quantify and track N transfer and validate the technique of infusing enriched $^{15}N_2$ into grass and legume root zones using H pots. The hypotheses tested were: 1) N resources can be shared between grasses and legumes through root contact; 2) annual ryegrass (*Lolium multiflorum* Lam.) may associate with microorganisms and reduce N_2 when enriched with $^{15}N_2$; 3) it is possible to enrich plants by injecting $^{15}N_2$ gas in the root zone; and 4) biologically fixed N exchange can be tracked in grass-legume mixtures using H pots.

Material and Methods

Experiment 1 – Single-pot Experiment

The experiment was conducted in a greenhouse environment arranged in a randomized complete block design in a 2 × 2 factorial arrangement (grass or legume species, with or without $^{15}N_2$ enrichment) (Fig. 1) with four replicates at the University of Florida - North Florida Research and Education Center (NFREC), Marianna, FL, USA (30°51’06”N, 85°09' 55”W, 35 m asl). ‘Prine’ annual ryegrass (*Lolium multiflorum* L.) and ‘Dixie’ crimson clover (*Trifolium incarnatum* L.) were the forage species, with treatments based on the use (or not) of enriched ^{15}N gas (98% atom ^{15}N - Sigma-Aldrich).

A soil mixture composed of 1/3 sand, 1/3 potting soil, and 1/3 field soil was made for inclusion in the pots. The potting mixture had a density of 0.725 g cm^{-3}. Average Mehlich- I extractable soil P, K, Mg, and Ca concentrations from air-dried soil sample were 68, 307, 336, 789 mg kg^{-1}. Estimated cation exchange capacity was 10.3 meq 100 g^{-1}, soil organic matter was 25.6 g kg^{-1}, and soil pH in water was 6.2. The ryegrass and crimson clover were planted in 4.2-L polyvinyl chloride (PVC) pipes on 13 January 2020 with one species on each pipe, using a seeding rate of 33.6 kg ha^{-1} and 28 kg ha^{-1}, respectively. Plants were watered every other day with 300 mL of water per pot. The bottom of the pots was filled with 2.4 kg of gravel to facilitate water drainage. Daily temperature ranged from 12.7 to 15.5°C with 15–20% relative humidity, and during the night, temperatures ranged from 7.2 to 10°C with 25 to 30% relative humidity. Each pot was sprayed with 14.8 mL of a Bifenthrin 2.4 mix (9.25 mL to 3.78 L of water) for spider mite control in March and April 2020.

A 0.3-cm diameter hole was drilled 16 cm from the top of each pot, into which an 18-cm long copper pipe was inserted to allow access to the root zone in the treatments receiving $^{15}N_2$ (Fig. 2, b). Holes were drilled in the copper tubes ~ 1-cm apart on their upper side to allow gas to flow directly into the root

zone. To this end, the pipes were fitted with a 2-way stopcock (Smith's Medical) attached to a 10-mL syringe (Becton Dickinson) (Fig. 2, c).

Two $^{15}N_2$ enrichment events took place. The first enrichment began on 16 April 2020 and the second on 7 May 2020. Every $^{15}N_2$ enrichment lasted 10 days, with 10 mL of the $^{15}N_2$ injected daily into the enriched treatments at a rate of 2 mL per hour from 1000 to 1400 (Fig. 4, b). Plants were staged to a 10-cm stubble using scissors on 9 April 2020, seven days before the first enrichment started (Fig. 4, a). The first harvest was on 30 April 2020, 14 days after the first enrichment, while the second harvest was on 21 May 2020, also 14 days after the second enrichment started. Herbage harvests occurred at 21-day intervals. Samples were dried in a forced-air oven at 55°C for 72 h after the harvest, and dry weights were recorded. All samples were ground to pass through a 2-mm screen using a Wiley Mill (Model 4 Thomas-Wiley Laboratory Mill; Thomas Scientific, Swedesboro, NJ), and analyzed for $\delta^{15}N$, N, and dry matter (DM) concentration.

Dry matter concentration was determined by weighing 1g of ground forage sample into crucibles and drying it at 105°C for 16 h. Carbon and N concentration were obtained through the Dumas dry combustion method using a Vario Micro Cube (Elementar, Hanau, Germany) after samples were ball milled utilizing a Mixer Mill MM400 (Retsch, Haan, Germany) at 25 Hz for 9 min. The Vario Micro Cube was coupled to an isotope ratio mass spectrometer (IsoPrime 100; Iso Prime, Manchester, UK) to determine the isotope ratio ($\delta^{15}N$). Biological N_2 fixation (BNF) was calculated using the technique described by Unkovich (2008). The proportion of plant N derived from the atmosphere (%Ndfa) was estimated using Eq. 1 described by Unkovich et al. (2008):

$$\%Ndfa = 1 - \left(\frac{atom\%^{15}NexcessN_2 - fixingplant}{atom\%^{15}Nexcessreferenceplant} \right) x100$$

Equation 1

$$\delta^{15}N\left(‰\right) = \left(\frac{sampleatom\%^{15}N - 0.3663}{0.3663} \right) x1000$$

Equation 2

where “atom%^{15}N excess N_2-fixing plant” is the atom% for the crimson clover found when using Eq. 2; and “atom%^{15}N excess N_2 excess reference plant” is the atom% for the reference plant when also using Eq. 2. The $\delta^{15}N$ for the crimson clover used for Eq. 2 was the $\delta^{15}N$ from the plant sample itself. The $\delta^{15}N$ for the reference plant came from the $\delta^{15}N$ obtained from an oat (*Avena sativa* L.) plant growing by itself in a separate pot without enrichment, and in the absence of inorganic N. Herbage BNF was estimated by multiplying the %Ndfa by the aboveground N accumulation, estimated by multiplying the herbage accumulation by the species N concentration.

Experiment 2 – H-Pot Experiment

The experiment was arranged in a randomized complete block design with four replicates in a greenhouse environment at the University of Florida - North Florida Research and Education Center (NFREC), Marianna, FL, USA. Sixteen-liter polyvinyl chloride (PVC) pipes connected in an H shape, referred to as H pots, were made using two PVC tees joined together with PVC coupling and medium blue PVC cement (Oatey Rain-R-Shine) to create an H shape, with PVC end caps on the bottom of each side. A 6-cm diameter hole was drilled in the middle of the horizontal portion of each H pot to facilitate uniform watering throughout all sections of the pot (Fig. 2, a).

'Prine' annual ryegrass (*Lolium multiflorum* L.) and 'Dixie' crimson clover (*Trifolium incarnatum* L.) were the forage species, with treatments based on the use (or not) of enriched ^{15}N gas (98% atom ^{15}N - Sigma-Aldrich), the ^{15}N gas infusion side (grass or legume), and the presence (or not) of a 60-µm mesh size synthetic membrane to reduce belowground interaction between grass and legume roots.

A 0.3-cm diameter hole was also drilled 16 cm from the top of each pot, into which an 18-cm long copper pipe was inserted to allow access to the root zone in the treatments receiving $^{15}N_2$ (Fig. 2, a). Holes were drilled in the copper tubes ~ 1-cm apart on their upper side to allow gas to flow directly into the root zone. To this end, the pipes were fitted with a 2-way stopcock (Smith's Medical) attached to a 10-mL syringe (Becton Dickinson) (Fig. 2, c).

For the first treatment (HCE), $^{15}N_2$ 98% atom was injected into the crimson clover side; in the second treatment (HGE), the $^{15}N_2$ gas was supplied to the annual ryegrass; in the third (H) treatment, none of the species received $^{15}N_2$; while in the fourth (HMCE), crimson clover received the $^{15}N_2$, however a 60-µm synthetic membrane was placed on the middle extremities of the pot to avoid root contact between the two species (HMCE) (Fig. 3).

The experiment was conducted in the same greenhouse environment under the same soil, temperature, humidity, planting, and enrichment conditions previously described for the first experiment. All plants were watered every other day, on both sides of the H pot and in the middle (H connection), with a total of 750 mL of water per pot. Biomass, $\delta^{15}N$, N, N accumulation, and DM were obtained similarly as described in Experiment 1.

Statistical Analysis

In both experiments, all response variables were analyzed using linear mixed model procedures as implemented in SAS PROC GLIMMIX (SAS/STAT 15.1, SAS Institute). Data from the two experiments were analyzed separately. Harvest, species, enrichment, and their interaction were considered fixed effects. Block was considered a random effect. The best covariance structure was selected based on the AICC fit statistic. Least square means were compared through pairwise *t* test using the PDIFF option of the LSMEANS statement. Differences were considered significant at $P \leq 0.05$. For the H pot experiment,

orthogonal contrasts were also performed to test for ‘membrane effect’ and ‘injection side effect’ using contrast statements.

Results

Experiment 1 – Single Pot Experiment

$\delta^{15}N$, Biomass, N, and N accumulation

There was an enrichment effect for $\delta^{15}N$ ($P < 0.0001$), with both species presenting greater $\delta^{15}N$ for the enriched treatment (42.5‰) than for the non-enriched (0.7‰) (Fig. 5). There was a harvest × species interaction for biomass ($P = 0.023$) because crimson clover biomass was greater for the second harvest (11 vs. 7 g pot^{-1}), while there was no difference between the harvests for annual ryegrass (1.4 and 2.0 g pot^{-1}, for 1st and 2nd harvests) (Table 1). Nitrogen concentration was affected by species ($P < 0.0001$) and harvest ($P = 0.0026$) (Table 1). Crimson clover had greater N concentration (34 g kg^{-1}) than annual ryegrass (12 g kg^{-1}). There was a harvest × species interaction ($P = 0.0137$) for N harvested, with greater N harvested in the second harvest for crimson clover (355 vs. 261 mg pot^{-1}) but no difference for annual ryegrass between Harvest 2 and Harvest 1 (22 and 21 mg pot^{-1}, respectively) (Table 2).

Table 1
Species effect and harvest effect for N concentration in the single pot experiment.

Species	N (g kg^{-1})
Crimson clover	34
Annual ryegrass	12
P value	< 0.0001
SE	0.8
Harvest	N (g kg^{-1})
1	25
2	22
P value	0.0026
SE	0.8

Table 2
Harvest × species interaction for biomass in the single pot experiment.

Harvest	Species	Biomass (g pot^{-1})	N accumulation (mg pot^{-1})
1	Crimson clover	7 ^{B*}	261^{B*}
	Annual ryegrass	1.4 ^{C}	21^{C}
2	Crimson clover	11 ^{A}	355^{A}
	Annual ryegrass	2 ^{C}	22^{C}
P value		0.0045	0.0137
SE		0.5555	21.80

*Means withing a column followed by a common uppercase letter do not differ ($P \geq 0.05$) according to LSD.

Experiment 2 – H-pot Experiment

Herbage $\delta^{15}N$

The $^{15}N_2$ supply to roots induced ($P < 0.0001$) $\delta^{15}N$ enrichment for annual ryegrass, with the most enriched $\delta^{15}N$ in treatments HCE and HGE (23.0 and 24.4‰, respectively) and the most depleted for treatments H (1.4‰) and HMCE (2.4‰) (Fig. 6A). There was also a membrane effect ($P < 0.0001$) when contrasting treatment HCE versus HMCE, indicating that the 60-µm mesh membrane significantly reduced ^{15}N transfer between the compartments, likely because it prevented root contact between the two forage species.

There was a treatment effect for $\delta^{15}N$ in the crimson clover ($P < 0.0001$). Treatments HCE (21.2‰) and HMCE (26.8‰) were the most enriched followed by treatments HGE (4.2‰) and H (-0.4‰) (Fig. 6B). There was also an injection side effect ($P < 0.0001$) when comparing treatments HCE and HGE, but as expected there was no effect of the membrane on the clover $\delta^{15}N$ in the HMCE treatment.

Biomass, N, N accumulation, and BNF

There was no treatment effect for any of the response variables, but total biomass was greater in the second than the first harvest for both annual ryegrass ($P = 0.0017$) and crimson clover ($P < 0.0001$). Annual ryegrass had a harvest effect for N concentration and N accumulation ($P < 0.0001$, $P = 0.0175$), while N concentration was greater in the first harvest (19 g kg^{-1}) than in the second (16 g kg^{-1}; Table 3). The greater biomass in the second harvest resulted in greater N accumulation (64 mg pot^{-1}) than in the first (36 mg pot^{-1}). There was no effect of harvest on crimson clover N concentration, but the greater biomass in the second harvest led to both greater N accumulation and BNF in the second harvest (Table 3).

Table 3
Nitrogen concentration and N accumulation of annual ryegrass and crimson clover and clover biological N_2 fixation (BNF) in two harvests in H pots.

Harvest	Annual ryegrass N, g kg^{-1}	Crimson clover N, g kg^{-1}	Annual ryegrass N accumulation, mg pot^{-1}	Crimson clover N accumulation, mg pot^{-1}	BNF clover, mg pot^{-1}
1	19	37	36	372	367
2	16	36	64	511	498
P value	< 0.0001	0.2635	0.0175	0.0010	0.0016
SE	1	1.3	8	41	44

Discussion

Experiment 1 – Single pot

The enrichment in $\delta^{15}N$ observed in the aboveground biomass for both species when the respective root systems received $^{15}N_2$ gradually in the root zone confirms the effectiveness of this technique to evaluate BNF even when the legume and the grass were growing separately. It is possible that certain bacteria associated with annual ryegrass may have a correlation with the presence of $\delta^{15}N$ in the biomass (Castanheira et al. 2014). Labeled N_2 must first undergo reduction before plant assimilation. As a result, further research is required to isolate and identify the microorganisms that are associated with N_2 reduction in annual ryegrass.

Additionally, enrichment is achieved at a significantly lower cost due to the much-reduced $^{15}N_2$ demand in comparison with atmospheric liberation of this gas. Although supplying $^{15}N_2$ to the plant would generally be considered the gold standard for BNF estimates, this is usually unfeasible due to cost and logistical requirements (Unkovich et al. 2008), which this experimental setup minimizes. At the same time, the lack of enrichment in crimson clover not supplied with the gas also indicates that atmosphere enrichment in the greenhouse was likely minimal, even though the experimental setup did not block gas transfer between soil and atmosphere.

Experiment 2 – H-pot Experiment

When $^{15}N_2$ was supplied to crimson clover in treatments HCE and HMCE, annual ryegrass accumulated ^{15}N in its biomass in HCE but not on HMCE, the latter being when direct root contact was prevented by the 60-µm synthetic membrane. This suggests a mechanism of transfer associated with roots of the two species growing in proximity to each other. This could be explained by direct connection (McKell and Duncan 1969), bridging by mycorrhizae hyphae (Hamel et al. 1991a; Hamel et al. 1991b; 1992), through root exudates specially at early growth stages (Lesuffleur et al. 2013) or the transfer of ^{15}N via free-living

soil microorganisms (Fustec et al. 2010; Jalonen et al. 2009). The enrichment observed for HGE, where the gas was directly supplied to annual ryegrass roots, indicates BNF either by free-living diazotrophs (Fracetto et al. 2019) or by associative microorganisms already found in annual ryegrass (Castanheira et al. 2014). Biological N_2 fixation has been reported in other grass and cereal species (Boddey and Dobereiner, 1995). Dobereiner (1966) initially reported BNF within bahiagrass (*Paspalum notatum* Flügge) and estimated that its association with *Bradyrhizobium paspali* was responsible for ~ 6 kg N ha^{-1} yr^{-1} across bahiagrass pastures. Santos et al. (2019) further supported these results across several bahiagrass cultivars, with quantities fixed of ~ 9 kg N ha^{-1} yr^{-1}. The greater N accumulation and BNF observed for the second harvest is likely due to plants being better established, as also observed when harvesting annual ryegrass for silage production (Anaya-Ortega et al. 2009).

It is pertinent to note that the current technique still has room for refinement, and there exist both advantages and disadvantages associated with its use. The 10-day enrichment procedure was selected due to the dearth of studies that utilized $^{15}N_2$ to track belowground N transfer in mixed swards and because the gas was directly injected into the root zone to track its distribution. However, it is essential to mention that the use of $^{15}N_2$ is relatively costly, and there is an opportunity to improve the technique by perhaps reducing the number of days and the quantity of gas used, thereby optimizing the effective tracking of N while also being mindful of resources.

Conclusions

The infusion of enriched $^{15}N_2$ at 12-cm depth gradually and directly into the root zone utilizing H-format pots can be used as a technique to track N transfer in grass-legume mixtures and BNF in general. This novel methodology can also be used with other stable isotopes to improve our knowledge of belowground network and resource sharing with plant and microbial communities.

The H-pot technique with $^{15}N_2$ infusion directly into the root zone indicated that root contact and/or mycorrhizae network should also be considered as important pathways for belowground N transfer, although often just aboveground N transfer pathways are considered.

The ^{15}N was present in annual ryegrass biomass in both H and single pots, which indicates this grass species may fix atmospheric nitrogen. Further studies are necessary to identify the soil or endophytic microorganisms responsible for this fixation.

Declarations

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Competing Interests

The authors report no conflict of interest. The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Luana M. D. Queiroz: Conceptualization, Data curation, Methodology, Formal analysis, Investigation, Writing original draft, Writing-review & editing.

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Figures

Figure 1

Single-pot treatments. (a) Crimson clover in a single pot enriched; (b) ryegrass in a single pot non-enriched (b); (c) crimson clover in a single pot non-enriched; (d) ryegrass in a single pot enriched. All photos courtesy of author.

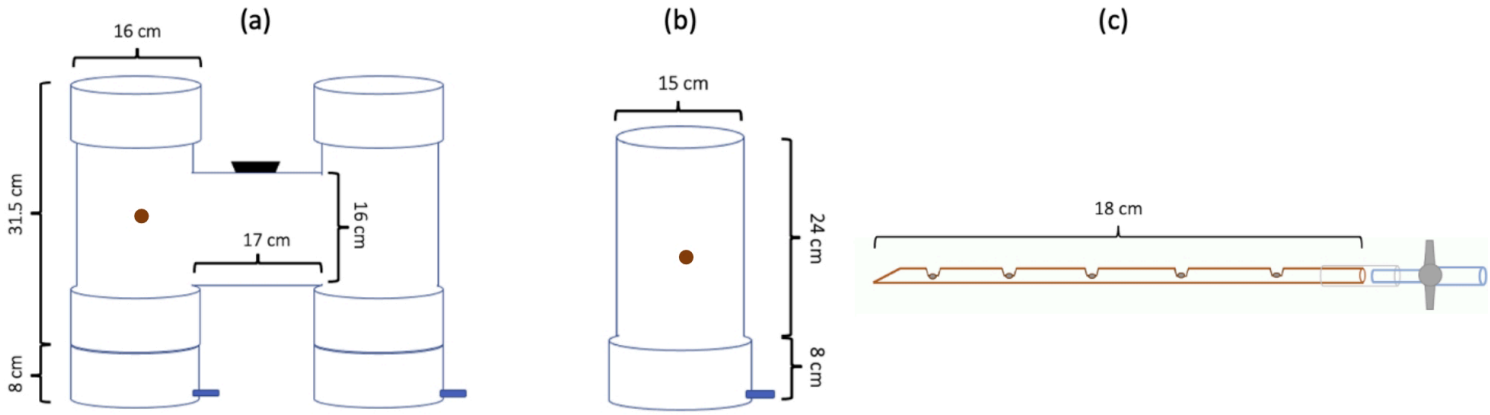

Figure 2

H pot (a) and single-pot (b) diagram with dimensions and perforated copper pipe (c) used to deliver $^{15}N_2$ to the root zone. On each pot type, the brown dot represents the point at which the copper pipes were placed. Figure drawn to the scale.

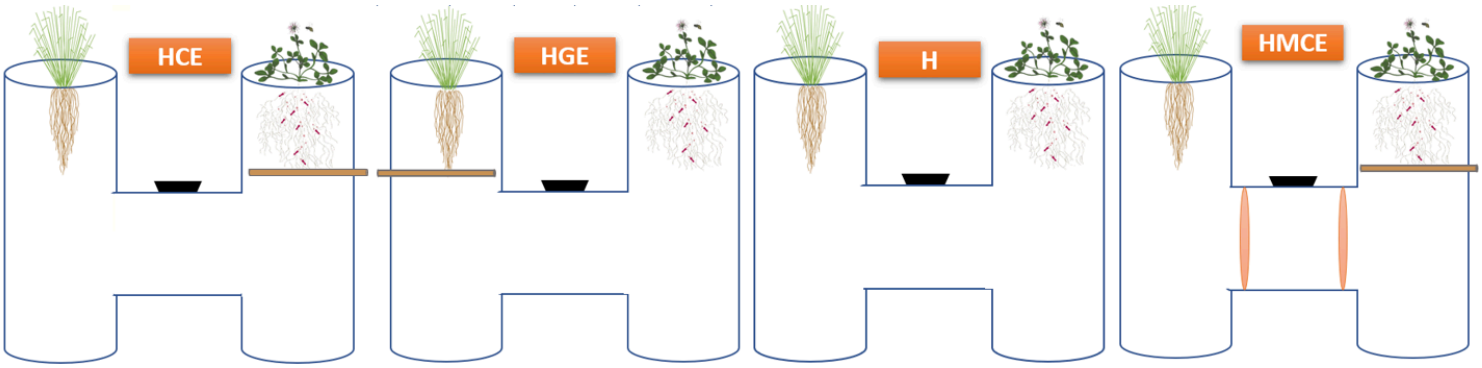

Figure 3

H-pot treatment layout. H pot in which the crimson clover side was receiving the $^{15}N_2$ 98% atom (HCE); H pot in which the ryegrass side was receiving the $^{15}N_2$ (HGE); H pot in which none of the species were receiving $^{15}N_2$ (H); and H pot in which the crimson clover was receiving the $^{15}N_2$, however a 60-µm membrane was placed (HMCE). Figures are drawn according to scale.

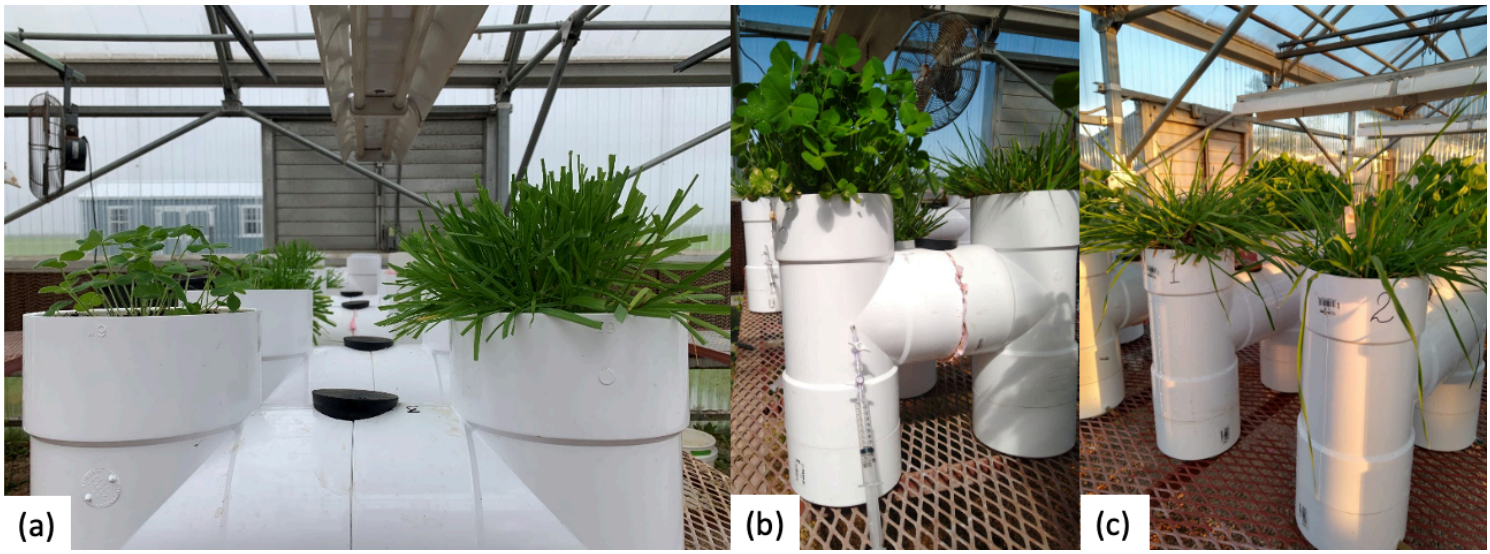

Figure 4

H-pot treatments. (a) Annual ryegrass and crimson clover plants after staging to 10 cm, one week before the enrichment started. (b) The $^{15}N_2$ was injected into the rootzone with a syringe through a copper tube containing a valve. (c) Annual ryegrass and crimson clover plants before the harvest, 14 days after enrichment. All photos courtesy of author.

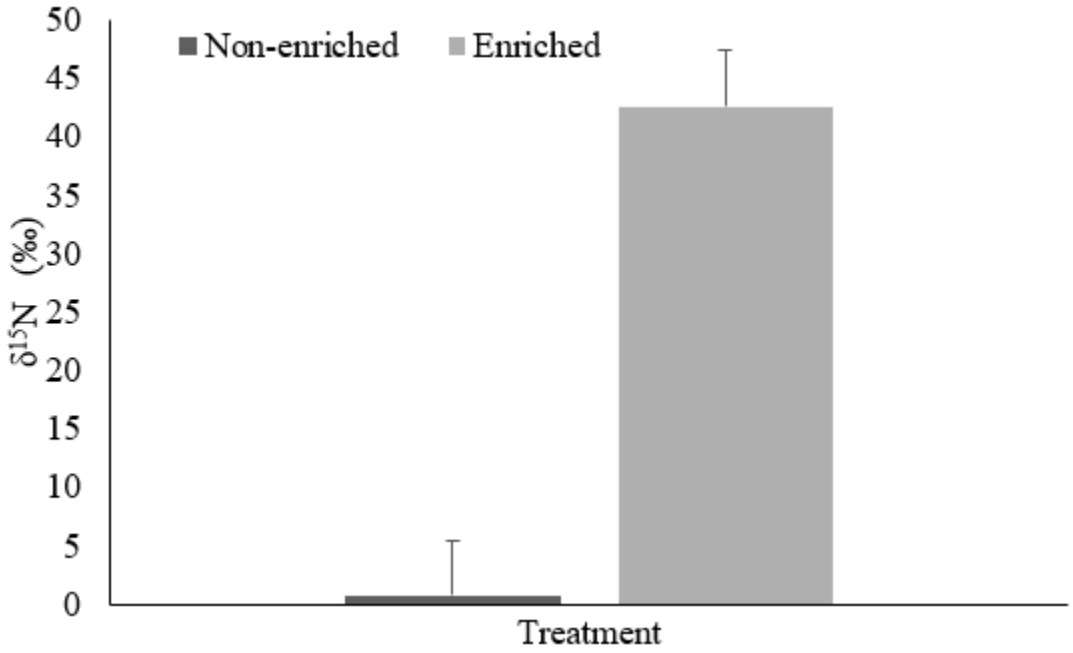

Figure 5

Enrichment effect ($P < 0.0001$) for the single pot experiment averaged across species, harvests, and replications.

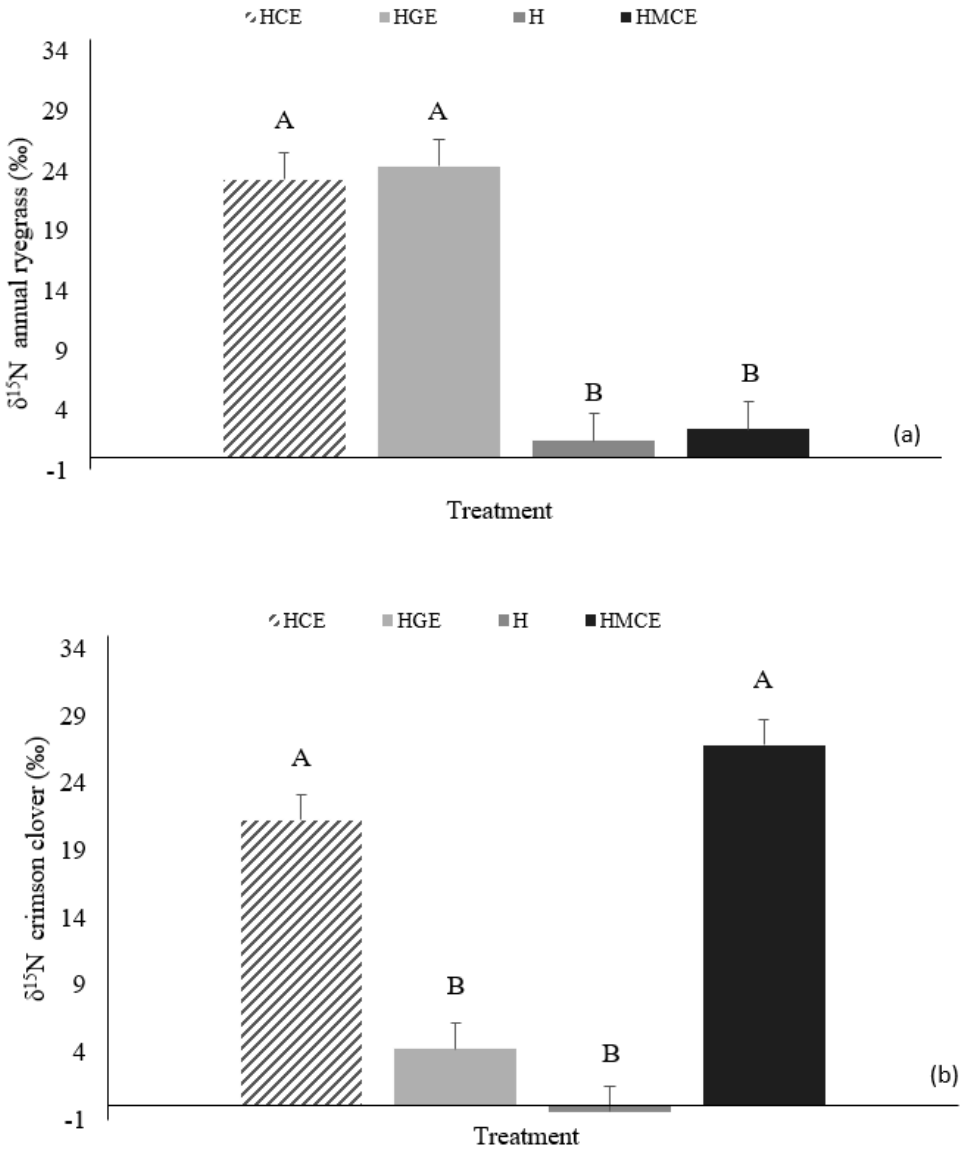

Figure 6

Treatment effect *(P* < 0.0001) for $\delta^{15}N$ in the ryegrass (a) and crimson clover (b) in H-pots. Treatments were: H-pot with the clover side enriched (HCE); H-pot with the grass side enriched (HGE); H-pot without enrichment (H); H-pot with the clover side enriched containing a membrane (HMCE). Means followed by a common uppercase letter do not differ (*P* ³ 0.05) according to LSD.