Evaluating the importance of a potential source of error when applying shoot ^{15}N labelling techniques to legumes to quantify the below-ground transfer of nitrogen to other species

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Abstract
Different ^{15}N-based shoot-labelling protocols have been utilised to quantify the below-ground transfer of N between pasture or crop species. The most straight forward and effective ^{15}N-labelling technique appears to be shoot labelling via leaflets or petioles. This paper reports a time-course study following ^{15}N-shoot labelling of pasture legumes to ascertain whether a pulse-feeding of ^{15}N labelled urea to the leaf resulted in induced exudation of highly ^{15}N enriched compounds from the roots into the surrounding soil. Such an occurrence could confound interpretation of ^{15}N data used to measure transfer of N between legume and non legume species. Small quantities of highly enriched ^{15}N (99.8 atom% ^{15}N) urea were fed to leaves of subterranean clover (*Trifolium subteraneum*; 0.22ml/plant of 1% urea solution) and lucerne (*Medicago sativa*; 0.45mL of 0.5% urea solution) over a period of 3 days. On days 1, 2, 4, 8, 16 and 32 after labelling, the plants were harvested and the ^{15}N enrichment of shoots, roots and soil was determined. Soil analyses showed that the ^{15}N enrichment of soil N was significantly higher in the lucerne treatment (12.3 ‰; parts per thousand ^{15}N relative to the ^{15}N content of atmospheric N_2, 0.3663 atom% ^{15}N) than subclover (11.3 ‰) ($P<0.05$), and both levels were significantly higher than, but very close to, the background level in the soil (10.7 ‰). The results could be explained if <10% of the lucerne root N or 12-27% of the subclover roots remained unrecovered in the soil following the sieving process. It was concluded that pulse-feeding of high concentrations of ^{15}N labelled urea in the leaf did not induce substantial exudation of highly ^{15}N enriched compounds from the roots into the surrounding soil.

Key Words
Subterranean Clover, Lucerne, N transfer, ^{15}N shoot labelling

Introduction
Different ^{15}N-based shoot-labelling protocols have been used to study the deposition of N-rich compounds and decaying root material in the plant's rhizophere (often described as rhizodeposition). Usually plants are ^{15}N-labelled during early growth and the label (and distribution of N) quantified later in shoots, roots and root-zone soil (e.g. McNeill *et al*. 1997; Khan *et al.* 2002). A key requirement of this approach is that the root-zone soil should not be directly labelled with ^{15}N. In other words, any ^{15}N in the soil should be of plant origin.

Shoots have been labelled by exposure to an atmosphere containing $^{15}NH_3$ or $^{15}N_2$ (single or multiple pulses) However, there is a level of sophistication and complexity in the equipment used in atmospheric labelling studies that detract from using this approach, particularly for field research (Russell and Fillery 1996). Another approach to ^{15}N labelling involves exposing part of the root system to a ^{15}N solution and recovering the label in the shoot and the other non-exposed part of the root (i.e. split root system) (Jensen 1996). Direct shoot labelling has also been used with the ^{15}N being applied either as a foliar spray, by immersing attached leaflets or petioles in vials containing ^{15}N enriched solutions, or by introducing enriched solution into the plant's stem (Høgh-Jensen and Schjoerring 2000; Khan *et al.* 2002; Russell and Fillery 1996; Zhou *et al.* 1998).

All three approaches, and the various labelling techniques within those approaches, have been developed for particular purposes and have their own distinct advantages and limitations. It is unlikely, therefore, that any one technique will be broadly applicable to all plant species. Some of the techniques require complex and/or expensive equipment (e.g. gas-tight enclosures), some have specific plant morphological requirements (e.g.

woody or hollow stems), while others result in the substantial disturbance of the plant-soil system (split-root technique) or pose potential risks of soil contamination via run-off from foliage of applied ^{15}N (spray application). The simplest technique appears to be ^{15}N-labelling via leaflets or petioles. It has been used in both glasshouse and field studies involving both large crop plants and small pasture species (Jensen 1996; Khan *et al.* 2002; McNeill *et al.* 1997). The objective of this study was to examine the time-course of ^{15}N-shoot labelling to ascertain i) whether a pulse loading of high concentrations of ^{15}N labelled urea in the leaves of subclover (*Trifolium subteraneum*) and lucerne (*Medicago sativa*) resulted in any potential artefacts such as induced exudation of highly ^{15}N enriched compounds from the roots into the surrounding soil and ii) whether ^{15}N shoot labelling is a viable technique for estimating the direct N transfer between legumes and non-legumes.

Methods

Soil was collected from the 0 – 15 cm depth interval of a Red Chromosol soil (Isbell 1996) derived from granite located on the Charles Sturt University farm, Wagga Wagga. The equivalent of 2.15 kg of oven dry soil was added to 3 L PVC pots by a standardised packing procedure to give an approximate bulk density of 1.3 Mg/m^3. Pots were watered to 90 % field capacity and 120 mL of basal fertiliser treatments were applied evenly to the soil surface. The basal fertiliser treatment ensured all nutrients, except N, were not limiting. Three plants of lucerne and subclover were established and maintained until shoot labelling, 5 weeks after emergence.

An earlier experiment was used to determine optimum urea concentration for subclover (1% w/w) and lucerne (0.5% w/w), which also revealed that lucerne absorbed approximately double the solution compared to subclover (0.25 vs 0.50 mL/leaf). The shoot labelling technique described by Khan *et al.*, (2002) was carried out on 1 leaf from each plant (3 leaves/pot). In brief, the selected leaflets were fully submerged in deionised water and the tips cut off with a razor. Excess water was removed with a tissue from the leaflets and the entire leaf was immediately inserted into a 2 mL Eppendorf vial containing 0.25 mL (1% w/w ^{15}N labelled urea solution 99.8 atom % ^{15}N excess) and 0.50 mL (0.5% w/w ^{15}N labelled urea solution 99.8 atom % ^{15}N excess) for subclover and lucerne, respectively. Blu-Tack was used to keep the vial in place while also sealing the top of the vial to prevent evaporative losses and attach the vial to a small wooden stake placed adjacent to the leaf. Tin-foil was placed on the soil surface beneath the fed-leaves to prevent any accidental spills of ^{15}N-enriched urea from contaminating the soil and remained in place to protect the soil surface until leaf feeding was complete. All Eppendorf vials were removed after 72 hours and weighed to determine the quantity of solution absorbed. Fed leaves and petioles were removed 24 hours after feeding to minimise risk of directly enriching the soil and were not included in any plant analysis.

The duration of the experiment was 32 days with a total of 6 sampling times, each sampling time had 4 replicates. Sampling of pots occurred at 1 day after ^{15}N labelling, 2 days later, 4 days later, 8 days later, 16 days later and at the completion of the experiment, 32 days after ^{15}N labelling. Throughout the experiment all fallen leaves were collected during plant growth to minimise the direct ^{15}N contamination of the soil, and later included in shoot analysis.

Sampling occurred by removing plant shoots at the soil surface. Root and soil material was passed through a series of sieves down to 1 mm. The collected root material was the gently washed free of remaining soil. All plant material (root and shoot) for each species was dried individually in a plant dehydrator at 80°C for 48 hours and dry matter yield (DMY) determined. Plant samples were then coarsely ground using a Cyclotec sample mill, before, being finely ground to a powder with a puck and ring grinder. The total N content and ^{15}N enrichment of the dried samples were determined by combustion using an automatic N and C analyser interfaced with a 20-20 stable isotope mass spectrometer. A representative sub sample of soil collected for total N content and ^{15}N enrichment and dried at 40°C for 48 hours before being finely ground to a powder with a puck and ring grinder. Soil samples were analysed as per the plant samples.

Results

Subclover had significantly ($P<0.05$) higher (approximately 2 to 3 times more) shoot dry matter yield than lucerne at each sampling time (Table 1). Lucerne had significantly ($P<0.05$) greater root biomass than subclover at each of the sampling times (Table 1). Shoot dry matter accumulated exponentially for both lucerne and subclover from 1 to 32 days after shoot labelling, whereas the increase in root dry matter was much more gradual for both species (Table 1).

Table 1. Shoot and root dry matter yield (g) for lucerne and subclover 1, 2, 4, 8, 16 and 32 days following shoot labelling with ^{15}N (99.8 atom% ^{15}N) urea. Shoot or root data designated with a different letter represents a significant difference (*P*<0.05).

Days after labelling	Shoot Dry Matter (g)		Root Dry Matter (g)	
	Subclover	Lucerne	Subclover	Lucerne
1	22.4 cd	7.3 f	13.5 d	17.8 c
2	20.3 d	8.1 ef	13.9 d	18.4 c
4	22.1 cd	8.4 ef	13.9 d	21.2 bc
8	25.2 c	8.9 ef	15.5 cd	22.1 bc
16	31.1 b	11.8 e	17.0 cd	24.8 b
32	52.2 a	22.7 cd	19.3 c	27.3 a
lsd (*P*=0.05)	4.3		3.9	

The ^{15}N enrichment of lucerne shoots was significantly (*P*<0.05) greater than the ^{15}N enrichment of subclover shoots, ranging from approximately 350 to 2110 ‰ higher (Table 2). Furthermore, ^{15}N enrichment of lucerne shoots declined significantly (*P*<0.05) at every sample time beyond 2 days after shoot labelling, whereas enrichment of subclover shoots remained the same across all sampling times (Table 2). The ^{15}N enrichment of roots was approximately 20 % of what was measured in the shoots (Table 2). The ^{15}N enrichment of lucerne roots was also significantly greater than the ^{15}N enrichment of subclover roots but unlike the lucerne shoots, the ^{15}N enrichment remained similar up until 8 days after shoot labelling before significantly (*P*<0.05) declining (Table 2). Root ^{15}N enrichment of subclover roots remained unchanged at all sampling times (Table 2).

Table 2. Shoot and root 15N enrichment (‰) for lucerne and subclover 1, 2, 4, 8, 16 and 32 days following shoot labelling with ^{15}N (99.8 atom% ^{15}N) urea. Shoot or root data designated with a different letter represents a significant difference (*P*<0.05).

Sampling Time (days)	Shoot ^{15}N Enrichment (‰)		Root ^{15}N Enrichment (‰)	
	Subclover	Lucerne	Subclover	Lucerne
1	315.4 e	2624.6 a	54.1 c	321.9 ab
2	342.7 e	2514.3 ab	60.4 c	326.9 a
4	358.5 e	2093.4 b	95.3 c	336.8 a
8	412.0 e	1581.2 c	86.9 c	340.0 a
16	258.4 e	878.3 d	91.2 c	262.4 b
32	101.4 e	450.7 de	74.0 c	220.6 b
lsd (*P*=0.05)	428.3		62.9	

There was no significant (*P*<0.05) change in ^{15}N enrichment of soil between any of the sampling times after shoot labelling. However, when averaged across all sampling times ^{15}N enrichment of soil from lucerne (12.3 ‰) pots was significantly (*P*<0.05) greater than subclover soil (11.2 ‰), while both were significantly (*P*<0.05) greater than the soil where no enriched plants were grown (10.6 ‰) (Figure 1).

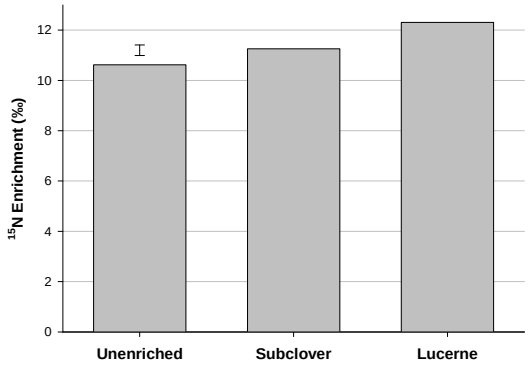

Figure 1. The ^{15}N enrichment (‰) of soil either unenriched or with subclover or lucerne grown. lsd bar (0.4) represents a significant difference where P<0.05.

Discussion

The large increases in shoot dry matter over the 32 day period for lucerne resulted in a significant dilution of the ^{15}N enrichment. Dilution of ^{15}N enrichment was also observed for subclover shoots, but not to the same extent. There was little change in the ^{15}N enrichment for either lucerne or subclover roots, which is most likely a reflection of the minor changes in root dry matter during the 32 days of the experiment. Despite the subclover and lucerne receiving equivalent quantities highly enriched ^{15}N (99.8 atom% ^{15}N) urea (2.9 and 3.0 mg/plot) the enrichment levels varied between the 2 species. Recovery of fed ^{15}N was 87 and 34 % for lucerne and subclover, respectively. The ^{15}N recovery observed for the lucerne was typical of the results reported by others (Khan *et al.* 2002; McNeill *et al.* 1997) but the recoveries for subclover were low. For both lucerne and subclover the labelled leaves and petiole (3 per pot) were not included in the ^{15}N analysis of shoots. Therefore, it is possible that a large majority of the unrecovered ^{15}N was not translocated from the labelled leaves and petiole. However, it was recognised that there was potential for losses associated with shoot labelling of urea as previous studies have identified that hydrolysis in the fed leaf can be exacerbated by high concentrations of urea-N. The subclover treatments in this experiment were labelled with 1 % (w/w) urea solution, which was double that of the lucerne treatments and may have an increased risk of hydrolysis losses in the fed leaves. The 1 % (w/w) urea solution concentration was selected following a preliminary study which indicated that subclover was not as sensitive to urea concentration as lucerne, but tended to take up only half the volume of solution. Ammonia could also be lost from the shoots which could be a source of error, although this ammonia might subsequently be reabsorbed by adjacent leaves or plants which could negate or create another source of error. The potential for these error sources in this study cannot be discounted.

Despite the low ^{15}N recovery for subclover and the potential for some losses there was still sufficient enrichment of both lucerne and subclover plant material to determine whether a pulse-feeding of ^{15}N labelled urea to the leaf resulted in induced exudation of highly ^{15}N enriched compounds from the roots into the surrounding soil. Soil analyses showed that the ^{15}N enrichment of soil N was significantly higher in the lucerne treatment (12.3 ‰) than subclover (11.3 ‰), and both levels were significantly higher than, but very close to, the background level in the soil (10.6 ‰). Based on the ^{15}N enrichment achieved the results could be explained if <10% of the lucerne root N or 12-27% of the subclover roots remained unrecovered in the soil following the series of sieving process that occurred at harvest to separate soil from roots. If it is assumed that 100 % of root material was recovered, an almost impossible task, then 1.3 and 1.4 % of recovered ^{15}N (0.4 and 1.1 % of total fed ^{15}N urea) was potentially exuded into the soil from subclover and lucerne, respectively.

Conclusion

It was concluded that pulse-feeding of high concentrations of ^{15}N labelled urea in the leaf did not induce substantial exudation of highly ^{15}N enriched compounds from the roots into the surrounding soil. The most probable explanation for the presence of small quantities ^{15}N in the soil would be associated with difficulty of completely removing root material from soil, particularly when roots are highly enriched. Therefore, shoot labelling via leaflets or petioles, which is a straight forward and effective ^{15}N-labelling technique, appears to be a suitable method to measure transfer of N between legume and non legume species.

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