Reassessment of the Contribution of Belowground N from Soybean after Testing Different 15N Leaf-Labelling Strategies

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Abstract

Purpose: Soybean is the most important grain crop in Brazil with a mean N accumulation of over 250 kg N ha^{-1}, principally from biological N_2 fixation. The residual N benefit depends heavily on the quantity of the belowground N at harvest, much which cannot be directly recovered in roots. The purpose of this study was to investigate different aspects of the ^{15}N leaf-labelling technique to quantify non-recoverable root N (NRRN) derived from senescent roots and nodules (rhizodeposits).

Methods: Soybean plants were grown in pots of soil and at 27 days after planting (vegetative stage V4) cut or whole leaves were exposed to highly enriched ^{15}N-labelled urea or glutamine. Seven sequential harvests of the plants and soil were taken until the final grain harvest at 70 days after labelling.

Results: After only 48 h, the plants labelled with ^{15}N urea transferred approximately 5% to the soil, while only 1% was found in the roots. Leakage of ^{15}N label was even more pronounced when the leaves were labelled with ^{15}N glutamine. After this initial leakage, the excess ^{15}N deposited in the soil only increased by a further 2.6% of applied label, which suggested that only part of this N represented senescence of roots or nodules.

Conclusions At the final harvest, N in roots separated from the soil amounted to 6.4% of total plant N. Discounting the early rapid deposition of ^{15}N-enriched N to the soil, our calculations indicated that at final harvest the total NRRN was 2.8% of total plant N.

Introduction

Soybean is quantitatively the fourth most important grain crop produced in the world after maize, wheat and rice when ranked by grain production (FAO-STAT- 2020). However, if ranked by protein production it is the world´s foremost crop. Brazil, the USA, Argentina and China are the main producers in that order. Herridge et al. (2008) estimated that globally soybean was responsible for over 77 % of all BNF contributions of all grain legumes. Soybean production has increased by over 60 % since the publication of that paper, far more than the production of other grain legumes, such that the proportion today must be over 80% (FAO-STAT, 2020). The quantification of the residual N left in the soil after harvest is essential to quantify the long-term sustainability of soybean crop rotations and also for the assessment of global fluxes of reactive nitrogen (Fowler et al. 2013).

In Brazil the proportion of N derived from air (%Ndfa) via BNF by soybean is estimated at approximately 80 % (Zotarelli et al. 2012). The harvest index of the cultivars at present planted in Brazil is estimated to be approximately 40 %. The grain is rich in nitrogen (approximately 6.5 % N – 37 to 41 % protein) and the remaining vegetative shoot tissue (stems, leaves and empty pods) is low in N content such that the harvest index (shoot tissue only) is estimated to be close to 80 %, as physically recovered roots at harvest contribute little to plant N (Alves et al. 2006; Zotarelli et al. 2012). It follows that even though the BNF inputs are large, with such a high harvest index there may be little overall addition of N to the soil for the next crop. This conclusion ignores the input of N derived from the roots present at time of harvest as well as other N deposited from senescent roots and nodules, exudates, lysates and sloughed off cells, collectively known as “rhizodeposits”. We use the term non-recoverable root nitrogen (NRRN) in this paper to include all N deposited in the soil which is not recoverable as roots by sieving the soil at harvest.

The amount of N deposited into the soil from roots until the time of harvest has generally been estimated with the use of the ^{15}N isotope (Wichern et al, 2008). Janzen and Bruisma (1989) were among the first to use the technique and labelled the shoots of wheat with ^{15}N-labelled ammonia gas. Since that time there have been many studies to estimate the quantity of non-recoverable root N (NRRN) of legumes and non-legumes using ^{15}N labelling of leaves, petioles, stems, “split-root technique” and other techniques (see Wichern et al. 2008). The split-root technique, and those that use gaseous forms of

^{15}N ($^{15}NH_3$, $^{15}N_2$ for N_2-fixing legumes), are generally impossible to use in the field. As our eventual objective was to find a technique to use on field-grown soybeans, the labelling of leaves, petioles or stems were possible choices.

As the petiole technique has been found less effective quantitatively for labelling the plants (McNeill et al. 1997; Yasmin et al. 2006), and the stem-feeding (cotton-wick) technique (Russell and Fillery 1996a) requires thick stems, the most frequently used technique is leaf-labelling generally following the "leaf-flap" procedure used by Khan et al. (2002). The technique relies on estimating the added labelled N (excess of ^{15}N above the natural abundance of the soil) and the ^{15}N enrichment of the tissue or compounds being lost by the plant to the soil. The roots are assumed to be the source of N lost to the soil so the calculation of the proportion (%) of N derived from the roots (%NdfR) becomes (Janzen and Bruisma, 1989):

%NdfR = (Atom%excess soil/Atom%excess root) x 100

To calculate the total N in the soil derived from rhizodeposition (NRRN), %NdfR must be multiplied by the total N in the soil (TN_{soil}).

NRRN = (%NdfR x TN_{soil})/100

As Janzen and Bruisma (1989) and several subsequent authors have stated (e.g. Rasmussen, 2011; Hupe et al. 2016), for the technique to estimate accurately the %NdfR, the following conditions or basic assumptions need to be met:

1. The N deposited in the soil has the same ^{15}N enrichment as the roots,
2. The added excess ^{15}N is evenly distributed in the root system,
3. The ^{15}N enrichment of the roots is constant over the growth period of the plants.

Most authors have labelled plants just once, so it is surprising that there have been so few studies on the uniformity of ^{15}N enrichment in the roots and changes in enrichment with time.

If plants are labelled with one pulse of a ^{15}N-labelled substrate at a relatively early stage of growth, as the plants grow and accumulate more nitrogen from the soil and/or, in the case of legumes, from BNF, it is to be expected that the ^{15}N enrichment of plant tissues will decline with time. This is generally observed for the shoot tissues of plants labelled in this manner, but surprisingly this does not always seem to be true for root tissues (McNeill et al. 1997; 1998; Gasser et al. 2015; Rasmussen et al. 2019). McNeill et al. (1997) labelled leaves of subterranean clover (*Trifolium subterraneum*) with ^{15}N-enriched urea at 42 days after planting. They found that the ^{15}N enrichment of the shoot tissue harvested decreased from 2.50 atom % ^{15}N excess 14 days after labelling (DAL) to 1.66 atom % ^{15}N excess 42 DAL, but root ^{15}N enrichment increased from 0.36 to 0.45 atom % ^{15}N excess in the same period and total N in the roots increased from 38 to 56 mg $plant^{-1}$. Similar results were registered for serradella (*Ornithopus compressus*). Their follow-up study with the same forage legumes showed that ^{15}N enrichment of roots of clover was precisely equal at 41 and 90 DAL, and the serradella roots presented only a small increase in ^{15}N enrichment from 0.73 to 0.79 atom % ^{15}N excess (McNeill et al. 1998).

Gasser et al. (2015) investigated the use of the leaf labelling technique to estimate the N derived from roots of red clover (*Trifolium pratense*). In this study leaf labelling of the plants was conducted at 83 days after germination and soil and plants were sampled starting 1 day after labelling and then at 14-day intervals for further 42 days. The ^{15}N enrichment of the root did not change significantly over the 42-day period. A further important result of this study was that the authors found that within one day of labelling a pulse of ^{15}N-enriched N was leaked into the growth medium (bentonite/sand).

If the ^{15}N enrichment of the roots remains approximately stable with time from leaf-labelling until final harvest, then the third basic assumption of Janzen and Bruisma (1989) that "The ^{15}N enrichment of the roots is constant over the growth period of the plants" would seem to be fulfilled. However, further studies are required to confirm this.

In preliminary studies by our team at Embrapa Agrobiologia with soybean we also noted that there was a considerable accumulation of ^{15}N-labelled N in the soil only three days after leaf-labelling with ^{15}N-enriched urea (Paredes et al. 2007). Root exudation induced by application of organic compounds to plant shoots was 'hot topic' many years ago, and exudation of amino compounds through roots was a known effect of spraying urea on plant shoot (Rovira et al. 1969). In addition, urea may freely cross plasmatic membranes owing to its size and nonpolar nature (Canarine et al. 2019), and then it can move from aerial tissues to the rhizosphere. It was thought that urea may be a substrate that caused considerable trauma to the plant metabolism owing to its rapid hydrolysis to ammonia which is toxic to the plant by several mechanisms (Gerendás et al. 2001), and that a compound such as glutamine might be a suitable alternative.

We hypothesised that:

1. If labelling plants with urea induces a short-term transfer (leakage) of enriched N into the soil, then ^{15}N-labelled glutamine will be less prone to cause this effect.
2. The estimates of "non-recoverable root nitrogen" (NRRN) will be independent of the source of ^{15}N-enriched N used for leaf labelling.

The objective of this study was to investigate the change in ^{15}N enrichment of shoot and root tissues of soybean and of the soil and the estimates of non-recoverable root N (NRRN) in the soil over time, when the plants were leaf-labelled with ^{15}N-labelled urea or glutamine.

Materials And Methods

Experimental design and planting

The experiment was conducted in a greenhouse of Embrapa Agrobiologia in Seropédica, Rio de Janeiro State. The soybean cultivar used was BRS 360RR of maturity group 6.2 with a growth cycle of between 104 and 129 days. The plants were grown in free-draining plastic pots containing 5 kg of soil (Acrisol – WRB/FAO classification) taken from the 0–20 cm depth interval of the soil from the Embrapa Agrobiologia field station. The soil was air dried and passed through a 2 mm sieve to remove roots and other debris and analysed using the standard techniques recommended by Embrapa (Claessen et al. 1997). The soil showed the following characteristics: pH 5.3, exchangeable cations (cmol kg^{-1}) Ca, 2.5; Mg, 0.5; K, 0.07; Al 0.0; available P (Mehlich 1), 1.0 mg kg^{-1}; total C (Walkley Black), 1.23 g kg^{-1}, total N 0.90 g kg^{-1}. Two weeks before planting the soil in each pot was thoroughly mixed with (per kg dry soil) 500 mg of lime, 100 mg P as single super phosphate, 66 mg K as potassium chloride and 50 mg of fritted trace elements. Three soybean seeds per pot inoculated with peat-based inoculant consisting of two *Bradyrhizobium* strains, SEMIA 5019 (29W – *B. elkanii*) and SEMIA 5080 (CPAC 7 – *B. diazoefficiens*) were planted on 22 October 2016. After 14 days the plants were thinned just one per pot.

The pots were laid out in randomized complete blocks with four leaf-labelling treatments, seven harvests and five replicates (blocks). The leaf-labelling treatments were: ^{15}N-labelled urea (ULL), ^{15}N-labelled glutamine (GLL) and ^{15}N-labelled urea + glucose (USLL) using the leaf-flap protocol and a further treatment with ^{15}N-labelled urea where an entire leaf was immersed in the labelled solution (UEL). The treatment with glucose was included as it was thought that its presence may enhance urea uptake and assimilation by the leaves (Borkowska and Szczerba, 1991).

Seven harvests were taken at 2, 7, 10, 14, 25, 47 and 70 days after leaf labelling (DAL) for the ULL treatment, coinciding with the growth stages V4, R1, R2, R3, R5, R6 and R7 (Fehr and Caviness, 1977). For the GLL treatment, six harvests were made, omitting that at 25 DAL, and for the USLL and UEL treatments harvests were made at 2, 7, 14 and 70 DAL only. A further set of 10 soybean plants which were not subjected to leaf labelling were included in the blocks to act as controls and harvested at 70 DAL.

The leaf labelling was performed at growth stage V4, 27 days after planting. For the leaf-flap protocol the central leaf of the last trifoliate, with leaves already expanded, was cut parallel to the central nerve permitting the insertion of the “lap” into an Eppendorf tube containing the labelling solution (McNeill et al. 1997). For the treatments ULL and USLL and UEL the labelling solution consisted of 1.0 ml of urea (5 mg mL^{-1}) enriched with ^{15}N at 95.5 atom % and in the case of the USLL treatment glucose was also added at 2.5 mg mL^{-1}. The labelling solution for treatment GLL consisted of 1.0 ml of glutamine (5 mg mL^{-1}) enriched with ^{15}N at 98.5 atom %. Only the amide N of the glutamine was ^{15}N labelled.

The leaves were fed for a total of 24 h after which the Eppendorf tube was removed and the volume of remaining solution estimated. The labelled leaf was severed after 14 days and stored for subsequent analysis for ^{15}N enrichment and total N. To avoid contamination of the soil surface with any labelled N the surface of the pot was covered with aluminium foil and irrigation was achieved by a tube passing through the side of the foil.

Harvests

For the first five harvests (2, 7, 10, 14 and 25 DAL) the shoot was severed at the soil surface and divided into first, second, third, fourth, fifth, sixth and seventh emergent leaves where present. For the sixth and seventh harvest (47 and 70 DAL) the whole shoot was harvested together. At all harvests the pots were inverted and the roots and nodules collected. All soil was weighed moist, and then passed sequentially through sieves of 2.0, 1.0, 0.5 and 0.125 mm to recover the maximum quantity of visible roots. All recovered roots were collected in plastic bags and frozen and then dried in a freeze-dryer to allow adhering soil to be separated from the roots (McNeill et al. 1997). This adhering soil was termed “rhizosphere soil” and was separately dried and weighed. The bulk soil was thoroughly mixed, and subsamples taken for estimation of moisture (dried at 105°C) and for N and ^{15}N enrichment (air dried at 65ºC).

The root systems were divided into primary, secondary and fine roots. All plant material was dried in a forced air oven (> 72 h at 65°C) and weighed. Roots and nodules were ground manually to a fine powder in a pestle and mortar with the aid of liquid nitrogen. All other plant material was initially ground with a Wiley mill (1.0 mm mesh) and then ground to a fine powder using a roller mill similar to that of Arnold and Schepers (2004). The samples of bulk soil and rhizosphere soil were likewise ground using the same type of roller mill.

Plant and soil analysis

Samples containing an estimated 30 to 50 µg N of all plant material, soil and rhizosphere soil were weighed into tin capsules for analysis of total N and ^{15}N enrichment with a continuous-flow, isotope-ratio mass spectrometer (a Flash 2000 total CN analyzer coupled to a Finnigan Delta Plus mass spectrometer - Thermo Scientific) in the “John Day Stable Isotope Laboratory” of Embrapa Agrobiologia.

Calculations

To estimate the quantity (mg) of excess ^{15}N in any compartment of soil or plant material the following equation was utilized:

mg $^{15}N = (N_{total}$ x atom % ^{15}N excess)/100 .....Eq. 01

where N_{total} is the total N content in mg and atom % ^{15}N excess is the ^{15}N enrichment – NA, where NA was the ^{15}N natural abundance of the compartment of unlabelled plants expressed as atom % ^{15}N. These values were taken from the unlabelled soil and parts of the ten unlabelled plants and were 0.3692, 0.3656, 0.3660 and 0.3690 atom % ^{15}N (equivalent to + 7.99, -1.98, -0.71 and + 7.41‰) for the soil, shoot, roots and nodules, respectively. To calculate the weighted mean atom % ^{15}N excess in the whole shoots and plants the mg of ^{15}N excess in each component was added and divided by sum of the total N contents (mg) of the components multiplied by 100 as described by Sanches Pacheco et al. (2017).

For the quantification of the non-recoverable root nitrogen (NRRN) in the soil (all N not recovered in roots and nodules), the equations of Janzen and Bruinsma (1989) described in the Introduction of were used:

$NRRN = TN_{soil} * (Atom\%excess\ soil/Atom\%excess\ root)$……. Eq. 02

Statistical analyses

Data were individually submitted to tests for normality of errors (Shapiro-Wilk) and homoscedasticity of variances by the Breusch-Pagan test. When data did not meet the assumptions of variance analysis, Box-Cox transformation was applied (Box and Cox, 1964; Osborne, 2010). Then data were submitted to analysis of variance (ANOVA). When ANOVA indicated significant differences, means of treatments were separated by the least significant difference (LSD) calculated by the t-test ($p < 0.05$). The software R-project version R 3.5.0 (R Development Core Team, 2018) with the packages lmtest (Zeileis and Hothorn, 2002) and ExpDes (Ferreira et al. 2013) was used for statistical analyses.

Results

Harvests were conducted at 2, 7, 14 and 70 DAL for all treatments, but also 10 and 47 DAL for GLL and 10, 25 and 47 DAL for ULL. To economise the need for ^{15}N analyses, the treatments USLL and UEL were only harvested on these four occasions to a), trace the behaviour of the ^{15}N-enriched N soon after labelling and b), to calculate a value of the NRRN at the final harvest.

There were no differences in accumulation of dry matter (DM) or nitrogen (N) of the soybean plants between the treatments with the four different leaf-labelling strategies (Figs. 1 and 2). The plant leaves were labelled 27 days after planting (stage V4) and the shoots continued to accumulate DM for the next 47 days after labelling (DAL) but in the final 23 days, shoot DM accumulation (until 70 DAP) essentially ceased (Fig. 1A). Nodule and root DM continued to increase until 47 DAP and then significantly decreased until final harvest (Figs. 1B, 1C). N accumulation of the shoot tissues increased rapidly after leaf labelling until 14 DAL but continued to increase at a lower rate until final harvest. This continued increase in N accumulation during the grain filling stage V5 to V6 (14 to 47 DAP) when nodule DM and N content were also increasing, indicated that N_2 fixation was still active. The final stage for V6 to V7 (47 to 70 DAL), the increase must have largely been to retranslocation of N from one plant tissue to another, as nodule mass decreased by 41 % and nodule total N by 68 % (Figs. 1C and 2C). Between 7 and 10 DAL, shoot N accumulation ceased or was negative, but N accumulation subsequently continued. This interruption of N accumulation may be attributed to the trauma induced by the leaf cutting and labelling with an exogenous source of N seven days earlier. There was no evidence that the labelling with glutamine was any less harmful than urea to N uptake in this 3-day period.

Nitrogen was lost from the roots from 14 DAL onwards: a total loss of 29 mg N $plant^{-1}$. Until 47 DAL nodules accumulated 2.23 g of DM and 71.5 mg N. In the last 23 days the N lost by the nodules amounted to 51 mg $plant^{-1}$.

The total excess ^{15}N fed to the leaves for the three treatments with 5 mg urea labelled at 95.5 atom % ^{15}N amounted to 2297 µg ^{15}N excess and for the 5 mg of glutamine (98.5 atom % ^{15}N) 500.2 µg ^{15}N excess. These were the amounts loaded in the 1 mL of solution and 100 % recovery by the plants is not to be expected. The ^{15}N enrichment of the shoot tissue increased rapidly after leaf labelling but at 10 DAL it started to decrease as plant growth and N accumulation diluted the ^{15}N-labelled N (Fig. 3). All root cohorts (primary, secondary and fine roots) increased in ^{15}N enrichment until 10 or 14 DAL, but nodules began to decline in ^{15}N enrichment after 7 DAL (Fig. 4). This was probably due to the rapid growth and N accumulation of the nodules after 7 DAL which is typical for soybean at this growth stage (34 days after planting). After 25 DAL the ^{15}N enrichments of the secondary and fine roots and nodules remained almost constant. However, the primary roots increased their ^{15}N enrichment until 47 DAL and at the final harvest those plants labelled with ^{15}N-enriched urea decreased in enrichment to values lower (0.202 atom % excess) than the other root cohorts (0.300 and 0.289 atom % excess), but still above that of the nodules (0.111 atom % excess). As the plants leaf-labelled with urea had been fed 4.6 times as much ^{15}N

excess as those fed with glutamine, it is to be expected that the ^{15}N enrichments of the shoot and roots would be approximately reduced by this factor. From 10 DAL onwards mean shoot ^{15}N enrichment of urea-labelled plants was 4.3 times greater than for those labelled with ^{15}N enriched glutamine. For the roots (weighted mean of all cohorts), this ratio was 4.6.

The ^{15}N enrichment of the rhizosphere soil was between 12 and 53 times higher than that of the bulk soil. Compared to the approximately 5 kg of bulk soil, the mass of rhizosphere soil was small. As root mass increased, the amount of rhizosphere soil increased, starting at 0.75 to 0.97 g plant^{-1} at 2 DAL, rising to between 4 and 7 g at the final harvest (70 DAL). The total enriched N deposited in the rhizosphere soil was on average only 2.2 % of that found in the bulk soil and less than 0.28 % of the total excess ^{15}N applied (Table 1).

Table 1

Recovery of applied ^{15}N excess in the soybean shoots, roots, soil and rhizosphere soil as a percentage of that applied as ^{15}N- labelled urea or labelled glutamine.

Treatment	Days after labelling						
	2	7	10	14	25	47	70
	Shoot						
Leaf-lap Ureia (ULL)	5.00# b	49.14 b	58.00	70.05	87,38	78.34	80.84
Leaf-lap Glutamine (GLL)	15.10 a	68.96 a	59.05	77.32		72.03	78.87
Leaf-lap Ureia + Glucose (USLL)	4.98 b	44.75 b		70.46			85.84
Entire leaf Urea (UEL)	5.42 b	44.04 b		74.09			79.83
CV (%)	7.8	11.7	13.4	12.5		35.1	19.0
	Root + nodule						
Leaf-lap Ureia	0.92 b	3.99	9.25	13.33 ab	8.35	12.50	4.54
Leaf-lap Glutamine	2.83 a	5.44	9.04	10.94 b		14.92	5.16
Leaf-lap Ureia + Glucose	0.82 b	3.66		10.70 b			4.50
Entire leaf Urea	0.95 b	3.55		14.66 a			4.35
CV (%)	17.7	23.7	13.7	15.7		11.4	12.4
	Soil						
Leaf-lap Ureia	3.93 b	5.77 b	5.97	6.01 b	3.99	8.01	9.26 b
Leaf-lap Glutamine	11.58 a	10.45 a	7.23	11.11 a		12.25	14.15 a
Leaf-lap Ureia + Glucose	5.22 b	4.76 c		6.28 b			6.62 c
Entire leaf Urea	4.59 b	5.72 b		11.58 a			5.83 c
CV (%)	1.8	23.4	16.1	19.7		60.9	1.5
	Rhizosphere soil						
Leaf-lap Ureia	0.06 b	0.20	0.18	0.14 bc	0.14	0.08	0.18
Leaf-lap Glutamine	0.15 a	0.22	0.17	0.23 a		0.11	0.17
Leaf-lap Ureia + Glucose	0.07 b	0.28		0.10 c			0.14
Entire leaf Urea	0.05 b	0.16		0.15 b			0.11
CV (%)	49.9	59.4	10.4	25.3		48.0	62.5
	Total						
Leaf-lap Ureia	9.96 b	59.10 b	73.40	89.54	99.85	99.01	94.82
Leaf-lap Glutamine	29.65 a	85.07 a	75.49	99.60		99.30	98.35
Leaf-lap Ureia + Glucose	11.09 b	53.46 b		87.54			97.08

Means followed by the same letter indicate that there was no significant effect ($P < 0.05$, LSD Student) for the same day after the labelling treatment on the % recovery of the ^{15}N excess label.

Treatment	Days after labelling					
Entire leaf Urea	11.01 b	53.47 b		100.49		90.13
CV (%)	1.9	5.3	10.9	9.8	27.1	16.0

[#] Means followed by the same letter indicate that there was no significant effect ($P < 0.05$, LSD Student) for the same day after the labelling treatment on the % recovery of the ^{15}N excess label.

The different methods of leaf labelling with ^{15}N-enriched urea had little impact on the accumulation of excess ^{15}N by the plants. At 2 DAL the recovery of applied label was approximately 5 % in the shoots and almost 1 % in the roots + nodules, with little effect of the addition of glucose (treatment USLL) or the use of entire intact leaves for labelling (treatment UEL) instead of the leaf-flap protocol (treatment ULL). For the treatments USLL and UEL, plants were harvested on only four occasions at 2, 7, 14 and 70 DAL. At these harvests, once again there was very little impact of the labelling treatment.

The plants labelled with ^{15}N-enriched glutamine absorbed the excess ^{15}N more rapidly than the urea labelled plants, reaching 15 % and 2.8 % of the applied label in the shoot and roots + nodules, respectively, at 2 DAL. After 10 DAL the recovery of applied ^{15}N label was statistically similar to the other treatments with urea. After this time, the quantity of excess ^{15}N in the soil gradually increased reaching at the final harvest (70 DAL) 9.4 and 6.8 % of the urea applied in treatments ULL and USLL, respectively. For the treatment where urea was applied to the entire leaf (UEL), the proportion of ^{15}N label (11.7 %) peaked at 14 DAL, and for the glutamine labelled treatment reached 14.3 % of applied label at the final harvest.

Two strategies were adopted to calculate NRRN. For the first (NRRN-A) it was assumed that all N deposited in the soil was derived from the fine roots and the ^{15}N enrichment of the fine roots was used as 'Atom%excess root' in Eq. 02 (Table 2). For the second strategy (NRRN-B) it was assumed that N deposited in the soil was derived from both the fine roots and the nodules and the weighted mean ^{15}N enrichment of roots and nodules was utilized. The estimates of NRRN for the harvests until 14 DAL are not reliable, because at this time the ^{15}N enrichment of the fine roots was changing rapidly (Fig. 4C) such that the enrichment of the N being deposited is unknown. However, the results do show that deposition of labelled N began very soon after leaf labelling and could not be considered to be gradual deposition of N from senescing roots and/or nodules.

Table 2

Total N in recovered roots and nodules and estimates of non-recoverable root N (NRRN mg plant^{-1}) utilizing A) the ^{15}N enrichment of fine roots, or B) the weighted mean ^{15}N enrichment of the fine roots + nodules as the sources of N deposited in the soil.

		Non-recoverable root N (NRRN)				Total belowground N	
Treatment	N in recovered roots and nodules	A) NRRN using fine roots as N source		B) NRRN using fine roots and nodules as N source		N in recovered roots + NRRN A)	N in recovered roots + NRRN B)
	mg	mg	%	mg	%	.....mg.....	
	2 Days after labelling						
Leaf-lap Urea ULL	24.3	162.4	54.2	114.8	45.7	186.7	139.2
Leaf-lap Glutamine GLL	23.1	139.9	49.64	103.3	42.8	163.0	126.4
Leaf-lap Urea + Glucose USLL	21.4	202.1	60.2	143.2	53.0	223.5	164.5
Entire leaf Urea UEL	22.7	206.2	56.9	113.0	46.8	228.9	135.7
	7 Days after labelling						
Leaf-lap Urea ULL	30.3	53.8 b	15.3 b[b]	45.1 b	13.3 b	84.1 b	75.4 b
Leaf-lap Glutamine GLL	31.3	113.3 a	27.8 a	67.1 a	19.1 a	144.6 a	98.5 a
Leaf-lap Urea + Glucose USLL	29.6	47.6 b	15.0 b	39.9 b	12.9 b	77.2 b	69.5 b
Entire leaf Urea UEL	26.8	53.4 b	16.1 b	45.1 b	14.0 b	80.2 b	71.9 b
	10 Days after labelling						

[a] Means followed by the same uppercase letters, or no uppercase letters, indicate no significant difference ($P < 0.05$, LSD Student) between the two procedures for calculating NRRN within the same leaf-labelling technique.

[b] Means followed by the same lowercase letters, or no lowercase letters, indicate no significant difference ($P < 0.05$, LSD Student) between means of the estimate of NRRN at the same sampling date.

		Non-recoverable root N (NRRN)				Total belowground N	
Leaf-lap Urea ULL	57.7	35.2	10.5	38.1	11.2	92.9	95.8
Leaf-lap Glutamine GLL	62.5	49.3	12.9	49.7	12.9	111.8	112.1
	14 Days after labelling						
Leaf-lap Urea ULL	83,4	37.3 c	8.2 c	39.3 c	8.6 c	120.6	122.7
Leaf-lap Glutamine GLL	80.3	86.8 a	17.6 a	89.0 a	17.9 a	167.1	169.3
Leaf-lap Urea + Glucose USLL	78.6	49.4 b	10.7 bc	47.6 bc	10.3 bc	128.1	126.3
Entire leaf Urea UEL	79.9	63.2 ab	12.7 b	63.7 ab	12.8 b	143.1	143.6
	25 Days after labelling						
Leaf-lap Urea ULL	77.5	36.5	6.6	39.2	7.1	114.0	116.7
	47 Days after labelling						
Leaf-lap Urea ULL	126.1	58.0	8.4 bB	82.4	11.5 A	184.1	208.5
Leaf-lap Glutamine GLL	124.7	86.3	11.9 a	103.2	13.8	211.0	227.9
	70 Days after labelling						
Leaf-lap Urea ULL	51.8	76.1 b	8.9 b	107.4 ab	12.1 a	127.5 b	158.8 ab
Leaf-lap Glutamine GLL	50.7	137.9 a	14.7 a	141.1a	15.0 a	188.6 a	191.7 a
Leaf-lap Urea + Glucose USLL	54.1	60.8 bc	6.9 b	81.5 bc	9.1 b	114.9 bc	135.6 b
Entire leaf Urea UEL	51.8	45.4 cB	5.3 cB	70.8 cA	8.1 bA	97.3 c	122.7 b
CV %	11.3	12.2	3.6	9.1	2.8	20.8	9.4

[a] Means followed by the same uppercase letters, or no uppercase letters, indicate no significant difference ($P < 0.05$, LSD Student) between the two procedures for calculating NRRN within the same leaf-labelling technique.

[b] Means followed by the same lowercase letters, or no lowercase letters, indicate no significant difference ($P < 0.05$, LSD Student) between means of the estimate of NRRN at the same sampling date.

The ^{15}N enrichment of the fine roots of the ULL treatment was relatively stable from 25 to 70 DAP, varying from 0.265 to 0.324 and then to 0.289 atom %^{15}N excess for the harvests at 25, 47 and 70 DAL, respectively (Fig. 4). Likewise, the

weighted mean ^{15}N enrichment of the fine roots + nodules varied from 0.246 to 0.228 and then to 0.202 atom %^{15}N excess for the same three harvests. The lower value of the ^{15}N enrichment of the fine roots + nodules compared to fine roots alone led to higher estimates of the NRRN based on roots + nodules at 70 DAL (Table 2). At this final harvest as well as those at 7, 14 and 25 DAL the estimate of NRRN was higher for the plants labelled with glutamine than in any of the urea labelling treatments. However, owing to the low proportion of labelled N in the glutamine, the ^{15}N enrichment of the bulk soil was very low (0.0008 atom % ^{15}N excess) and these estimates cannot be considered to be reliable.

When the N in the recovered roots and nodules was added to the estimate of the NRRN based on the ^{15}N enrichment of fine roots only (NRRN-A), the total N was estimated to be 128 and 115 mg N plant^{-1} for the ULL and USLL treatments, and significantly lower ($P < 0.05$) at 97 mg N plant^{-1} when intact leaves were labelled. When recovered root and nodule N was added to NRRN based on the ^{15}N enrichment of fine roots + nodules (NRRN-B) the total N was estimated to be between 123 and 191 mg N plant^{-1}. Using urea labelled plants, the estimates of the total N left in the soil if all shoot material was removed from the field ranged from 11.5 to 14.8 % and 13.3 to 16.9 % of the entire plant N, for the estimates based on NRRN-A and NRRN-B respectively.

Discussion

As was reported in the review of the literature of Wichern et al. (2008), the ^{15}N label is concentrated in the shoot tissue and in this study the amount of belowground excess ^{15}N in roots, nodules and soil did not exceed 25 % of that applied. A few studies have followed the change in ^{15}N enrichment of plant tissues and soil with time after labelling (McNeill et al. 1997, 1998; McNeill and Fillery, 2008; Gasser et al. 2015; Hupe et al. 2016; Rasmussen et al. 2019). As was recorded here (Fig. 3), all the studies showed that the aerial tissue decreased in ^{15}N enrichment with time after labelling, owing to the dilution of the fixed amount of enriched N with increasing unlabelled N derived from soil and/or BNF. However, root DM and N accumulation with time have been found to be slow or even negative. Root DM decreased after 47 DAL and total root N decreased from a peak of 62 mg at 14 DAL to 30 mg plant^{-1} at the final harvest (Figs. 1C and 2C). However, nodule DM accumulation was rapid from 25 to 47 DAL reaching 2.2 g at 47 DAL and decreased to 1.3 g plant^{-1} at 70 DAP (Fig. 1D). Nodule total N showed a similar behaviour (Fig. 2D). As the ^{15}N enrichment of the nodules changed very little with time, the N for nodule growth from 25 to 47 DAL (Fig. 4D) must have come from both labelled and non-labelled sources, the latter most likely being BNF. Many other studies have shown that nodules are lower in ^{15}N enrichment than roots (e.g. Oghoghorie and Pate, 1972; Jensen, 1996; Russell and Fillery, 1996b) but there seems to be no other reports of the change in ^{15}N enrichment of nodules with time.

The six papers cited where the ^{15}N enrichment of different root cohorts was followed with time all reported results similar to those presented here (McNeill et al. 1997, 1998; McNeill and Fillery, 2008; Gasser et al. 2015; Hupe et al. 2016; Rasmussen et al. 2019). Root ^{15}N enrichment, especially secondary or fine roots, varied little over time after two to three weeks after leaf-labelling. This is unexpected, but seems to be mainly due to very slow N accumulation of roots pre-established for 20 to 40 days and hence little dilution of the original fixed amount of excess ^{15}N. Our results also show that while N accumulated by nodules increased from 22 mg plant^{-1} at 25 DAL to 71 mg at 47 DAL and returned to 21 mg plant^{-1} at 70 DAL, the ^{15}N enrichment only varied from 0.150 to 0.159 to 0.111 atom % ^{15}N excess over these three sequential harvests.

This relatively low variation in ^{15}N enrichment of roots has been observed by all authors who made sequential harvests to study this. However, only in this study and those of Gardner et al. (2012), Gasser et al. (2015) and Rasmussen et al. (2019) was the soil sampled only one or two days after leaf-labelling. In all cases some of the applied ^{15}N-enriched N was found in the soil after only 24 or 48 h after leaf-labelling. Gardner et al. (2012) reported that 1.3 to 1.4 % of the enriched N was recovered in the soil, when sub-clover (*Trifolium subterraneum*) or lucerne (*Medicago sativa*) leaves were labelled with ^{15}N-enriched urea. Gasser et al. (2015) working with red clover (*Trifolium pratense*) found that 0.5 % of the excess ^{15}N was

recovered in the bentonite/sand mixture just 24 h after leaf-labelling. Rasmussen et al (2019), reported that the ^{15}N-enriched N deposited in the soil in the first 24 h after leaf-labelling with ^{15}N-enriched urea was equivalent to up to 5 % of unrecovered roots of white and red clover (*T. repens* and *T. pratense*, respectively). Gardner et al. (2012) suggested that the presence of ^{15}N label in the first day after labelling was due to unrecovered roots which were impossible to separate from the soil, but even after 32 days there was no significant increase in the excess ^{15}N recovered, which begs the question as to why there was no further increase in the quantity of unrecovered roots in the soil during this period. Rasmussen et al. (2019) also suggested that much of this short-term deposition of excess ^{15}N in the soil could be due to unrecovered roots, but they recognised that unrecovered labelled roots could not explain the rapid appearance of ^{15}N in neighbouring grass which corresponded to 6–8 % and 12 to 16 % of the excess ^{15}N recovered outside the labelled plant (soil + grass). In contrast the rapid loss of a small proportion of excess ^{15}N from red clover was attributed to leakage of soluble forms of ^{15}N by Gasser et al. (2015).

In our study on soybean in the treatments leaf labelled with urea, by 2 DAL approximately 5 % of the ^{15}N label had been transferred from the labelled leaf to the shoot and less than 1% was found in the roots. However, in this 48-h period the mean “leakage” to the soil was between 4.0 and 5.9 % of all labelled N, thus amounting to almost half of the N exported from the labelled leaf to the shoot, roots and soil (Table 1). There was a similar behaviour of the excess ^{15}N deposited in the soil derived from labelled glutamine. At 2 DAP, only 2.8 % of the applied N was recovered in the roots while 11.6 % was released into the soil. As the amount of ^{15}N excess in the roots of all treatments at 2 DAL was much lower than that found in the soil, the roots must be considered to be a conduit for enriched N rather than a source. This supports the hypothesis of Gasser et al. (2015) that this excess ^{15}N was lost from the roots in solution.

Rasmussen et al. (2019) strongly criticised the conclusion of Gasser et al (2015) that the excess ^{15}N found in the growth medium just 24 h after labelling was due to leakage of soluble forms of enriched N into the soil. However, in their own study they found enriched N in neighbouring grass roots just 24 h after leaf labelling. They attributed this to labelled N in “root exudates” which was clearly in solution for such rapid transfer. Gasser et al. (2015) included plants in their study which were not subjected to leaf labelling or any manipulation of the leaves. They showed that there was significantly more ammonium in the leaf-labelled plants, so that this increase in ammonium was definitely associated with the process of labelling the leaves. Rasmussen et al. (2019) suggested that the damage caused to the petioles during leaf-labelling may have been partially responsible of the short-term ^{15}N leakage. In our study in two of the treatments labelled with enriched urea (ULL and USLL) the leaves were cut (leaf-flap technique – Khan et al. 2002) but the amount of excess ^{15}N found in the soils after 24 h was not significantly different between these treatments and the treatment UEL where uncut leaf tips were immersed in the enriched urea solution.

It is logical to expect that the source of “rhizodeposits” should mostly be root exudates and products of root and nodule turnover and senescence. The expected chronological pattern would thus be a gradually increasing rate of loss of N from roots with time and especially considerable losses from senescent nodules. This was not the pattern observed in this study. The mean quantity of excess ^{15}N deposited in the soil in the first 7 DAL from the urea labelled plants was 5.5 % of that fed to the leaves. Subsequently, over the next 63 days, this only increased further by 1.8 %, amounting to a total on average of 7.2 % (Table 1). Thus, the results suggested that most of the total excess ^{15}N deposited in the soil at the final harvest came from initial leakage from the root, although some of this soluble labelled N could have been reabsorbed.

Labelling with ^{15}N-enriched glutamine appeared to have no advantages over ^{15}N-enriched urea. Initial uptake by the plant was faster but short-term deposition in the soil seemed more severe than was the case with the urea-labelling treatments. For the urea-labelling treatments, an average 4.6 % of the ^{15}N label was deposited in the soil in the first two days; for the glutamine, the amount was estimated to be 11.6 % and, unlike the urea, some of the label appeared to be re-absorbed over the next eight days such that at 10 DAP the amount of excess ^{15}N in the soil decreased to 7.2%.

Initially we utilized two strategies to calculate the NRRN both assuming that the sole sources of ^{15}N excess in the soil were fine roots and nodules. In the first strategy, A, the fine roots were assumed to be the sole source of the N loss and in the second strategy, B, fine roots and nodules were the source. The calculated values for the NRRN, A and B, were respectively, 76 and 107 mg N for the ULL treatment, 61 and 82 mg N for the USLL, and 45 and 71 mg N for the UEL treatment. The estimates for the UEL treatment (intact leaf labelling) were significantly lower ($P > 0.05$) than those using the leaf-flap technique (treatment ULL).

Few other authors have compared the effect of different labelling techniques on the estimates of NRRN. Khan et al (2002) investigated different concentrations of urea and different methods of labelling (petiole or leaf-flap) fababean (*Vicia faba*), chickpea (*Cicer arietinum*), mungbean (*Vigna radiata*) and pigeonpea (*Cajanus cajan*) but did not calculate values of NRRN. Other authors have also studied the efficiency and distribution of ^{15}N label in legume and plants (Merbach et al, 2000; Hertenberger and Wanek, 2004; Yasmin et al. 2006) but none of them made comparative estimates of NRRN in plants grown in soil. An exception was the study of Chalk et al. (2002) who found that the injection of labelled N into the hollow stem of *Sesbania rostrata* resulted in an estimate of NRRN of only 13 % of whole plant N compared to 42 and 56 % for leaf and root immersion, respectively. In this present study, the plants labelled with glutamine gave significantly higher estimates of NRRN than those labelled with urea.

There are three previous evaluations of NRRN for soybean. Rochester et al. (1998) performed a study in the field in New South Wales, Australia. The plants were labelled via the petiole three times at three-week intervals. At pod-filling stage the plant shoots accumulated 431 kg N ha^{-1} and belowground N (in recovered roots and NRRN) was estimated to be 275 kg N ha^{-1}. At maturity, total shoot N increased to 525 kg N ha^{-1} and RBGN was estimated to have decreased to 162 kg N ha^{-1}. This indicates that the RBGN at maturity was approximately 24 % of total plant N. However, their final conclusion was that approximately 40 % of total plant N (the belowground N present at pod-filling stage) remained in the soil and this estimate of 40 % for RBGN thus appears to be unjustified from the results from this study (Rochester et al. 1998).

A further study on soybean by Laberge et al (2009) was performed at two sites in the field in Nigeria, Ibadan in the West and Zaria in the North. Plants were leaf-labelled with urea twice within a three-week period. Soybean yields were not high, at 1032 kg and 1571 kg ha^{-1}, for Ibadan and Zaria, respectively. The NRRN was estimated to be 11.6 and 18.0 % of the total accumulated plant N, respectively.

Zang et al (2018) estimated the NRRN of soybean in China. The plants were grown in soil in pots in the greenhouse. They used the stem labelling (cotton wick) technique and added ^{15}N-enriched urea solution every two weeks after planting. One harvest was performed at grain maturity and they estimated that NRRN was 23.5 % of whole plant N.

The evidence in our study suggests that the excess ^{15}N derived from the labelled urea that was found in the soil in the first 2 to 7 days was due to leakage, or some form of ^{15}N deposition provoked by the leaf-labelling process, and not to root senescence or other rhizodeposition which would occur in undisturbed plants. In this case the amount of excess ^{15}N deposited in the soil from 7 DAL until the final harvest at 70 DAL falls from 166.1 µg to 40.5 µg excess ^{15}N $plant^{-1}$. Using these data, the estimates of NRRN-A and NRRN-B become 14.8 and 22.2 mg $plant^{-1}$, respectively, 1.9 and 2.7 % of total plant N. As loss of N from nodules was very significant, we assume that the estimate NRRN-B of 22.2 mg $plant^{-1}$ is more likely to be close to actual N loss to the soil. Even so, the proportion of N lost as NRRN (2.7 %) is almost an order of magnitude lower than the estimates of NRRN for soybean by Rochester et al (1998), Laberge et al (2009) and Zang et al (2018) that ranged from 12 to 24 % of total plant N. This estimate is also far lower than those for most other legumes using ^{15}N labelling of leaves or stems (Wichern et al. 2008; Fustec et al. 2010).

The total N in roots and nodules recovered from the soil by sieving amounted to a mean of 51.9 mg $plant^{-1}$. Adding this to the estimate of NRRN-B of 22.2, the total residual belowground N (RBGN) was 74.1 mg $plant^{-1}$, or 9.1 % of plant total N.

Soybean in Brazil in 2020 had a mean yield of 3,250 kg grain ha^{-1}. Assuming a typical plant density of 330,000 plants per ha and a concentration of N in the grain of 6.5 %, each plant would accumulate approximately 750 mg N in the grain (250 kg N ha^{-1}) and assuming a nitrogen harvest index (NHI) of 80 %, a total N accumulation of 935 mg (313 kg N ha^{-1}) is estimated. In this study the total N accumulated by the plants at the final harvest reached 800 mg which indicates that the plants were almost as large as in the average field crop. For the average Brazilian soybean crop the total residual belowground N after soybean harvest (RBGN) would amount to approximately 28 kg N ha^{-1} of which 20 kg N ha^{-1} is derived from recovered roots and 8.5 kg N ha^{-1} from non-recoverable root N (NRRN).

Conclusions

With respect to the hypotheses outlined in the Introduction, the labelling of the plants with ^{15}N-enriched glutamine appeared to induce more leakage of ^{15}N to the soil at 2 DAL than the labelling with ^{15}N-enriched urea. Furthermore, the use of labelled glutamine resulted in higher estimates of NRRN than labelled urea and the addition of glucose to the labelled urea resulted in significantly lower estimates of NRRN. Thus, the estimates of NRRN may not be independent of the labelling technique as we hypothesised.

Gasser et al (2015) recommended that the ^{15}N enrichment of roots should be monitored with time, and that a sampling of plants and soil should be taken soon after ^{15}N labelling of the plant to catch any early leakage of ^{15}N-enriched N. In such a short space of time it is inconceivable that roots or nodules of unlabelled plants would suddenly contribute significant amounts of rhizodeposits, such that we suggest that this initial release of labelled N should be discounted from the total rhizodeposit N. In the case of Gasser et al (2015) discounting of this early leakage of enriched N reduced the estimate of NRRN by 41 % (equivalent to a 70 % overestimate of uncorrected value). In this present study correcting for initial leakage reduced the estimate of NRRN by 74 % (equivalent to a 290 % overestimate of uncorrected value). Even the uncorrected value is considerably lower than from earlier studies on soybean and many estimates of NRRN from other legumes. However, from the results obtained, it is reasonable to assume that the initial stimulation of the deposition of ^{15}N-enriched N within the first two days after labelling would cause an overestimation of NRRN. The proportion of the excess ^{15}N that was leaked is considerably higher than that experienced by Gasser et al (2015). The difference could be due to the different species studied or the fact that in our study the plants were grown in soil where a larger microbial biomass may have enhanced N leakage by rapid assimilation.

We conclude that almost all estimates of non-recoverable belowground N published to date, are large overestimates and we echo the recommendations of Gasser (2015) that the ^{15}N enrichment of roots should be monitored with time, and that a sampling of plants and soil should be taken soon after ^{15}N labelling of the plant to catch any early deposition of ^{15}N-enriched N and to discount this amount of enriched N before calculating the NRRN.

Declarations

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Authors' contributions. The authors Karla Araujo, Carlos Vergara, Ricardo dos Santos and Wadson Menezes Souza all worked on the planting, leaf-labelling, and the harvests of the experiment. Karla Araujo was mainly responsible for sample preparation for the isotope ratio mass spectrometer and the computation of the results (first try). Claudia Jantalia, Bruno Alves and Segundo Urquiaga were the supervisors of Wadson Souza, Ricardo dos Santos and Karla Araujo respectively. Robert Boddey planned the experiment and protocols with Karla Araujo, Segundo Urquiaga and Bruno Alves. Robert Boddey was principally responsible for writing the final manuscript.

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Figures

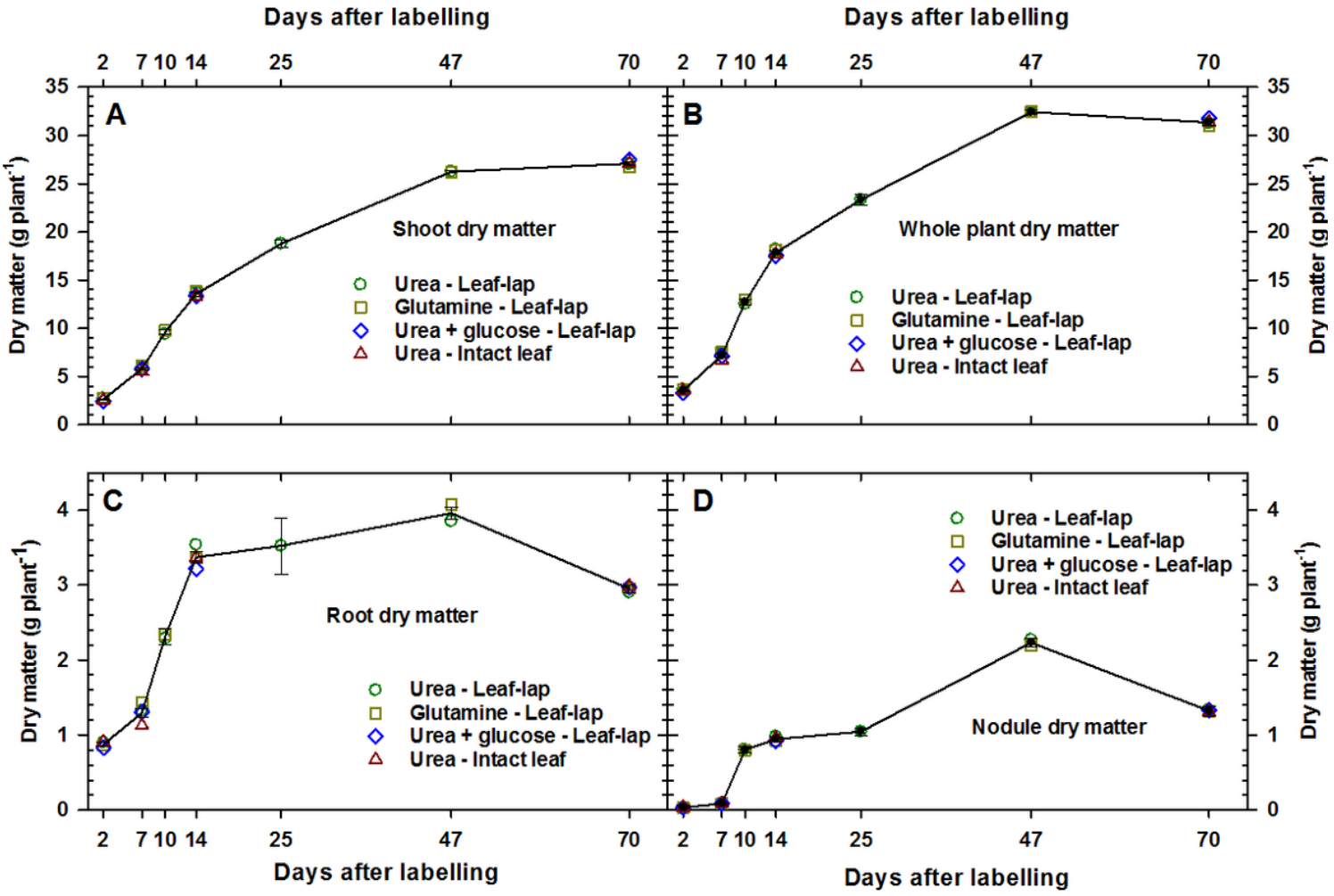

Figure 1

Dry matter accumulation by A shoots, B whole plants, C roots and D nodules and of soybean labelled with 15N-enriched urea or glutamine at 47 days after planting. Four different labelling methods were used, [O] 15N-labelled urea via leaf-flap, [O] 15N-labelled glutamine via leaf-flap, [♦] 15N-labelled urea mixed with glucose via leaf-flap and [▲]15N-labelled urea applied to intact leaves. Harvests were taken until grain maturity at 70 days after labelling (117 days after planting). Values are means of five replicates. Error bars indicate standard error of the means.

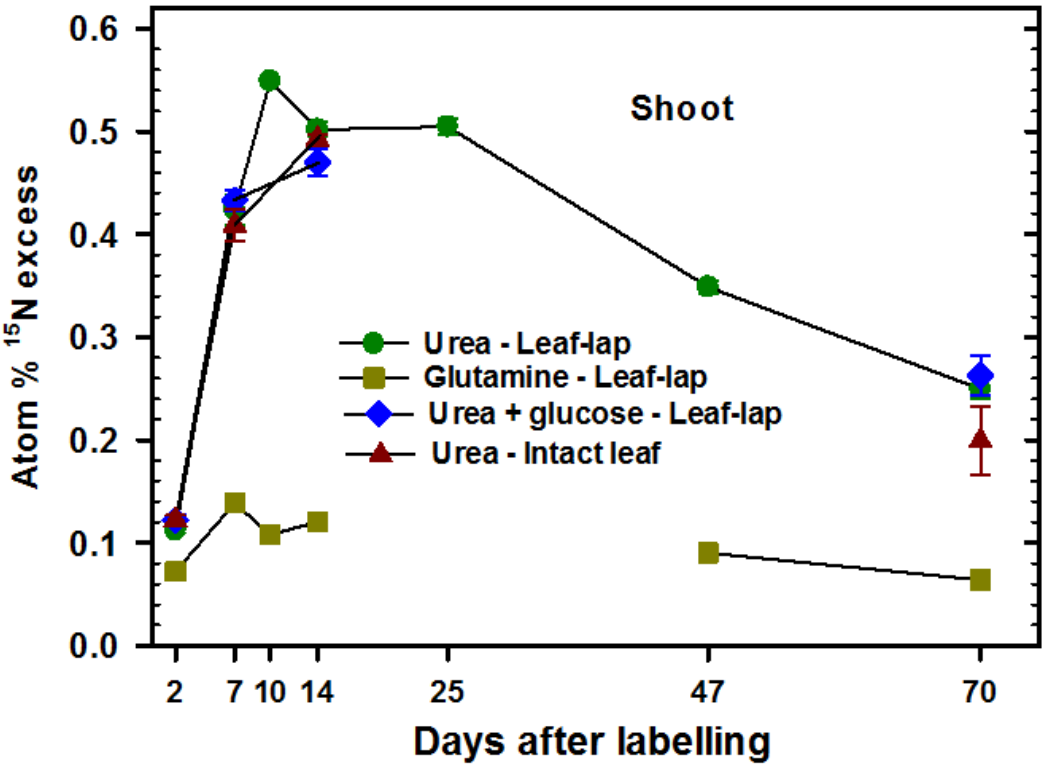

Figure 2

Nitrogen accumulation A shoots, B whole plants, C roots and D nodules and of soybean labelled with 15N-enriched urea or glutamine at 47 days after planting. Four different labelling methods were used, [O] 15N-labelled urea via leaf-flap, [O] 15N-labelled glutamine via leaf-flap, [♦] 15N-labelled urea mixed with glucose via leaf-flap and [▲]15N-labelled urea applied to intact leaves. Harvests were taken until grain maturity at 70 days after labelling (117 days after planting). Values are means of five replicates. Error bars indicate standard error of the means.

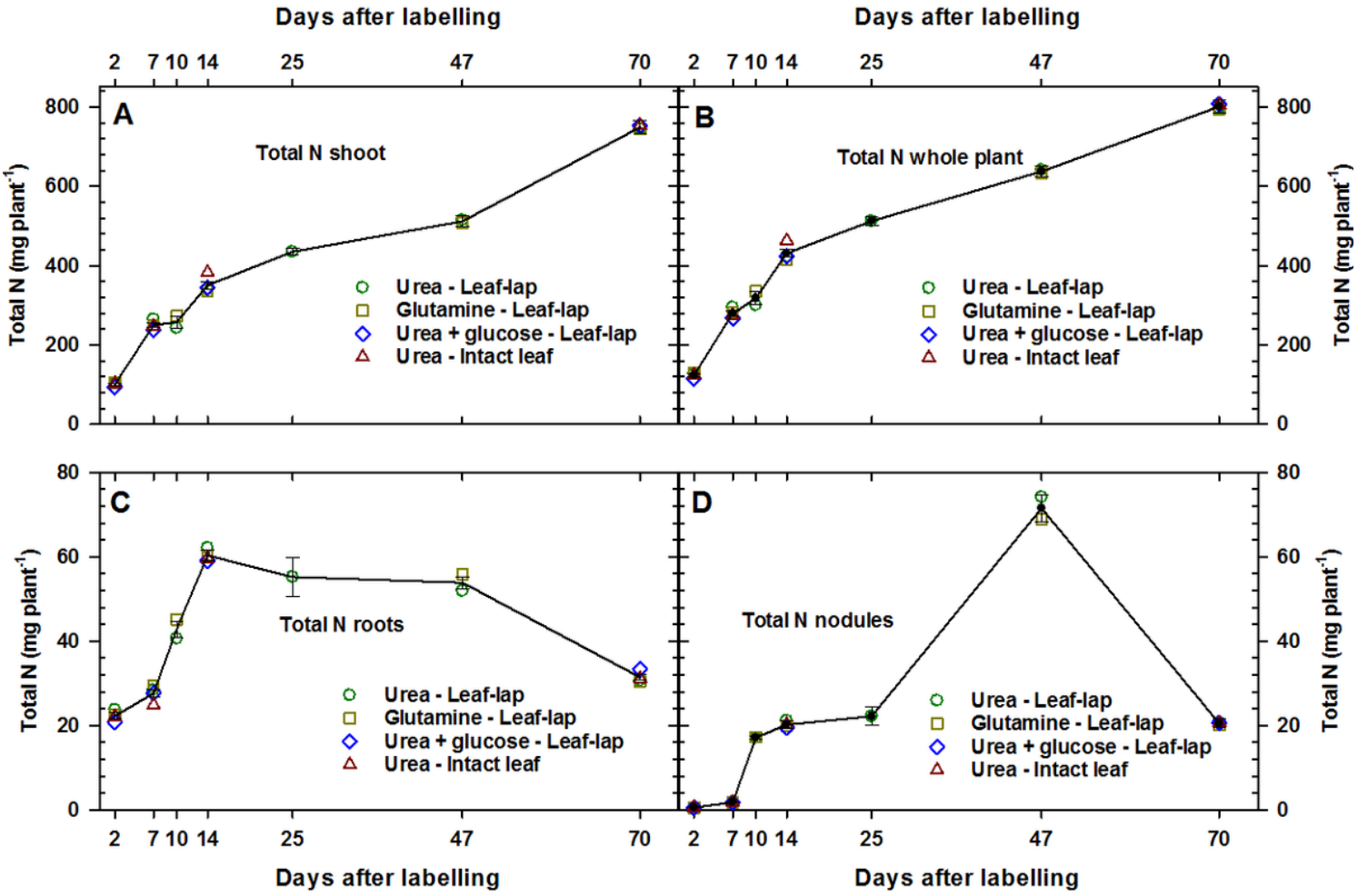

Figure 3

15N enrichment (atom % 15N excess) of shoots of soybean labelled with 15N-enriched urea or glutamine at 47 days after planting. Four different labelling methods were used, [O] 15N-labelled urea via leaf-flap, [O] 15N-labelled glutamine via leaf-flap, [♦] 15N-labelled urea mixed with glucose via leaf-flap and [▲]15N-labelled urea applied to intact leaves. Harvests were taken until grain maturity at 70 days after labelling (117 days after planting). Values are means of five replicates. Error bars indicate standard error of the means.

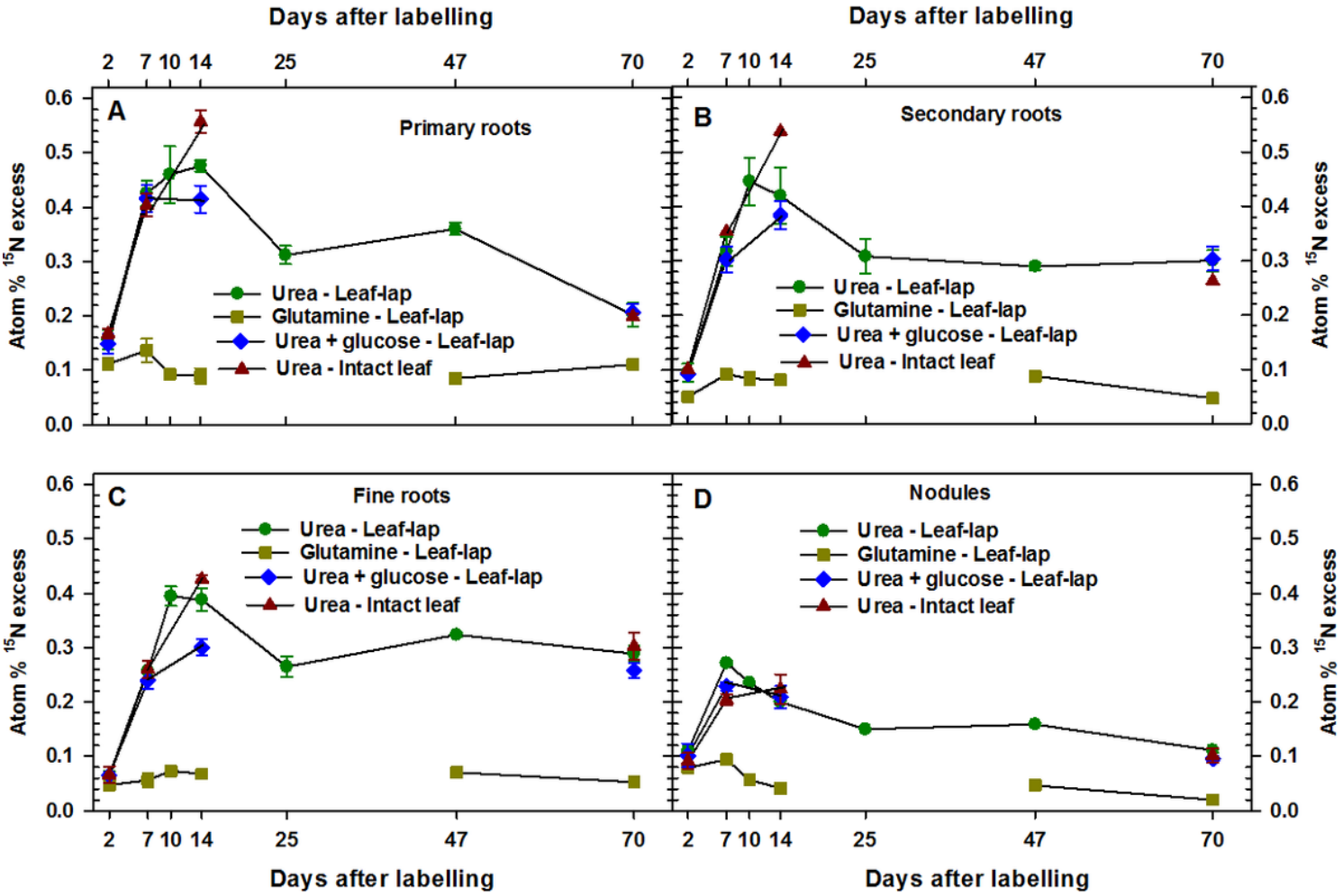

Figure 4

15N enrichment of different root cohorts (A primary, B secondary and C fine roots) and D nodules of soybean labelled with 15N-enriched urea or glutamine at 47 days after planting. Four different labelling methods were used, [O] 15N-labelled urea via leaf-flap, [O] 15N-labelled glutamine via leaf-flap, [♦] 15N-labelled urea mixed with glucose via leaf-flap and [▲]15N-labelled urea applied to intact leaves. Harvests were taken until grain maturity at 70 days after labelling (117 days after planting). Values are means of five replicates. Error bars indicate standard error of the means.

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