Nutr Cycl Agroecosyst
https://doi.org/10.1007/s10705-022-10238-w

ORIGINAL ARTICLE

Can ^{15}N leaf-labelling reliably quantify rhizodeposited nitrogen remaining after a nodulated legume crop?

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Received: 25 February 2022 / Accepted: 26 September 2022

Abstract Soybean is Brazil´s most important grain crop and accumulates 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 of which cannot be directly recovered in roots. The purpose of this study was to investigate different aspects of the ^{15}N shoot-labelling technique to quantify non-recoverable N in rhizodeposits. Three pot experiments were performed and the aerial tissue was labelled with highly enriched ^{15}N-labelled urea or glutamine at between 27 and 39 days after planting. In all experiments sequential harvests were taken until late grain-filling phase. After only 2 or 3 days between 5.8 and 21.3% of enriched N was found in the soil but the excess ^{15}N deposited until the final harvest was in all cases less than twice this amount, respectively. Evidence obtained suggested that this early deposition of labelled N was an artefact of the labelling technique. Discounting this initial tracer N decreased estimates of rhizodeposited N by between 51 and 66%. Nodules were much lower in ^{15}N enrichment than roots. Nodule N constituted 39 to 76% of belowground N, such that the inclusion of none or all of this N to calculate the ^{15}N enrichment of the roots increased the estimates of rhizodeposited N by between 34 and 58%. We conclude that even if the immediate post-labelling deposition of enriched N is discounted, estimates of rhizodeposited N of nodulated legumes will not be reliable.

Keywords ^{15}N mass balance · Belowground N · Nodule N · Residual N · Rhizodeposits · Soybean

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10705-022-10238-w.

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Published online: 26 November 2022

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 2022). 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 and contributes over 77% of all contributions of biological N_2 fixation (BNF) to grain legumes (Herridge et al. 2008). 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 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% (Hungria et al. 2006; 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 tissues (stems, leaves and empty pods) are low in N content such that the nitrogen harvest index (shoot tissue only) is estimated to be close to 80%, as physically recoverable roots at harvest contribute little to plant N (Alves et al. 2006; Zotarelli et al. 2012). So, 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".

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; Wang et al. 2020). Janzen and Bruinsma (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 rhizodeposits 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 difficult to use in the field. Our long-term objective was to quantify N rhizodeposition by soybeans in the field so 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, usually following the "leaf-flap" procedure used by Khan et al. (2002a). 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. Janzen and Bruinsma (1989) assumed that roots are the source of N lost to the soil, so the calculation of the proportion (%) of soil N derived from rhizodeposition (%NRhSN) becomes:

$$\%\mathrm{NRhSN} = (\mathrm{Atom}\%\ \mathrm{excess\ soil}/\mathrm{Atom}\%\ \mathrm{excess\ root}) \times 100$$

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

$$\mathrm{NdfRh} = \left(\%\mathrm{NRhSN} \times \mathrm{TN}_{\mathrm{soil}}\right)/100$$

As Janzen and Bruinsma (1989) and several subsequent authors have stated (e.g. Rasmussen 2011; Hupe et al. 2016), for the technique to estimate accurately the percentage of plant N deposited in the soil (%NdfRh), 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.

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

In recent studies several authors have used an alternative method to that of Janzen and Bruinsma (1989) to calculate rhizodeposited-N: the "isotope mass balance" technique. This technique was described in

detail by Hupe et al. (2016). The first difference is that the isotope ratios are not calculated as ratios of numbers of atoms (as in atom % ^{15}N and atom % ^{15}N excess), but as masses of the atoms. This makes a small (~7%) but constant difference to methods based on atom ratios. The second difference is that in this technique as formulated by Hupe et al. (2016), the source of labelled rhizodeposited-N is assumed to be the entire plant. This latter assumption was stated by Hupe et al. (2018) as: (i) the distribution of the tracer in the plant corresponds to the distribution of total N, (ii) the isotopic enrichment of roots and rhizodeposits is equal in space and 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 rhizodeposition by 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 roots 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 found in the growth medium (bentonite/sand). Rasmussen et al (2019) similarly observed ^{15}N excess derived from leaf-labelled clover plants in the soil and in neighbouring ryegrass plants within 24 h of labelling.

In the first 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 1969). In addition, urea may freely cross plasmatic membranes owing to its size and nonpolar nature (Canarini 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. 1997), and that a compound such as glutamine might be a suitable alternative.

Most authors have assumed with a single-pulse of labelling of the leaves or stems the ^{15}N enrichment of the roots will decrease with time as accumulation of non-labelled N diluted the fixed amount of added enriched N (e.g. Mayer et al. 2003; Mahieu et al. 2007; Wichern et al. 2008; Hupe et al. 2016). Several authors have suggested that repeated labelling over time could mitigate this decrease but there a few studies which have used multiple labelling and followed the changes in ^{15}N enrichment of roots over time (Hupe et al. 2016, 2018, 2019). Hupe et al (2016, 2018) found that multiple labelling could maintain ^{15}N enrichment approximately constant with time, but if there was an immediate short-term deposition of enriched N into the soil as observed in our preliminary study and those of Gasser et al (2015) and Rasmussen et al (2019), this could not be determined as no harvests were made immediately after the first stem labelling. Our study was therefore restricted to the investigation of possible ^{15}N deposition into the soil immediately after a single leaf labelling and the subsequent changes in the ^{15}N enrichment of roots over time.

A further problem with the technique may arise with nodulated root systems. Many reports on rhizodeposition of N from grain legumes do not

mention the existence of nodules (e.g. Mayer et al. 2003; Wichern et al. 2007a, 2007b; Mahieu et al. 2007; Hupe et al; 2016; 2018). However, nodules are rich in N and in shoot-labelled plants always lower in ^{15}N enrichment than roots (e.g. Oghoghorie and Pate 1972; Jensen 1996a, b; Russell and Fillery 1996a; Khan et al. 2002a). To apply the Janzen and Bruinsma technique it is necessary to know the ^{15}N enrichment of the roots. If the roots are nodulated then the value of the ^{15}N enrichment will be between that of the roots and the nodules. The objective of labelling the shoot with ^{15}N-enriched N is to obviate the need of complete sampling of the belowground biomass, so the question of what proportions of N are derived from nodules and clean roots becomes a source of error in the application of the technique.

The objectives of this study were to investigate after a single pulse labelling with a ^{15}N enriched substrate:

A. the changes in ^{15}N enrichment of shoot and root tissues of soybean and of the soil over sequential harvests,
B. to compare the estimates of rhizodeposited-N (NdfRh) made using different ^{15}N-labelled substrates for labelling and different assumptions for the calculations.
C. to investigate the potential magnitude of the error introduced by incomplete sampling of the nodules and roots.

We hypothesised that:

1. If labelling plants with urea induces a short-term leakage of enriched N into the soil, then ^{15}N-labelled glutamine will not cause this effect.
2. The estimates of rhizodeposit-N calculated using the ^{15}N enrichment of roots of the labelled plants as the source of this N (Janzen and Bruinsma 1989), will be higher than estimates made using the isotope mass balance technique (Hupe et al. (2016) which assume that the source of this N is the whole plant (weighted mean atom % ^{15}N excess).
3. A large proportion of belowground N of soybean is in the form of nodules, which are considerably lower in ^{15}N enrichment than roots. Failure to obtain a representative sample of the nodulated roots will introduce an error to the estimates of rhizodeposited N.

Materials and methods

Three pot experiments with soybean were performed in the greenhouse at Embrapa Agrobiologia. The soil used to fill the pots was topsoil (0–20 cm) of an Acrisol (WRB/FAO classification) taken from an area of the field station which had never been planted to soybean and was known from previous studies (Guimarães et al. 2008; Pauferro et al. 2010) to not possess significant numbers of rhizobia capable of nodulating this crop. Details of pot size, soybean variety, fertilizers and rhizobium inoculants applied for the three experiments are given in Table 1. The soil for each experiment was taken from the same area but not at the same time. The results of the analyses of the soil used for each experiment were not identical, so that the amount of lime and fertilizers added to the pots differed somewhat.

Table 1 Details of pot size, soybean variety, fertilizers and rhizobium inoculants applied in the three experiments

Experiment	Pot size (kg soil)	Soybean variety	Lime (g kg^{-1})	Potassium	Phosphorus	Trace elements	Rhizobium inoculant
					(mg kg^{-1})		
01	4	cv. Celeste	1	50	30	30	[a]SEMIA 5019, SEMIA 5080
02	8	cv. Celeste	1	50	45	30	SEMIA 5080
03	5	BRS 360RR	0.5	66	100	50	[b]SEMIA 5019, SEMIA 5080

[a]Applied as two different inoculants, one for each treatment

[b]One inoculant composed of a mixture of the two rhizobium strains

Experiment 1: Effect of different Bradyrhizobium strains on N in rhizodeposits.

For this experiment forty pots were destined to be leaf-labelled with ^{15}N enriched urea, half of them were inoculated with a peat-based inoculum made with the *Bradyrhizobium elkanii* strain 29 W (SEMIA 5019), and half with the *B. diazoefficiens* strain CPAC 7 (SEMIA 5080).

A further eight pots were simultaneously sown to soybean, four inoculated with SEMIA 5019 and four with SEMIA 5080 but these plants were not subsequently leaf labelled with ^{15}N-enriched N and used solely to quantify the contribution of biological N_2 fixation (BNF) applying the ^{15}N natural abundance technique (Shearer and Kohl 1986). To act as non-N_2-fixing reference plants for this satellite experiment, four pots each were planted to dwarf sorghum (*Sorghum vulgare* cv. BR 310), rice (*Oryza sativa,* cv. IAC 4440) or the common weed known as tropical spiderwort, or "trapoeraba" in Brazil (*Commelina benghalensis*). All pots, those with soybean destined for leaf labelling and those not labelled, and the non-legume reference plants were randomly allocated to a complete block design with four replicates.

In all pots, three seeds were planted per pot and nine days later seedlings were removed to leave just 1 plant per pot. Before leaf labelling, the soil surface was protected with aluminium foil and irrigated via a rubber tube to avoid possible leaching of ^{15}N-enriched N from the plants into the soil. This protection was maintained until the end of the experiment.

The soybean plants were leaf labelled at 35 days after planting as follows: One leaf at the centre of the most-recently fully-expanded tri-foliate group of leaves of all plants was dosed with ^{15}N labelled urea as described by McNeill et al. (1997). The leaf was cut twice longitudinally at each side of the central nerve to form a narrow flap which was immersed 1 ml of an aqueous solution of 5 g L^{-1} of ^{15}N-labelled urea (99.5 atom % ^{15}N) in an Eppendorf tube. The addition amounted to 2.385 mg ^{15}N excess in both treatments. The tube was supported by a galvanised wire with the lower end of the wire inserted into the soil, and a loop at the top fixed to the Eppendorf using adhesive tape. After 3 days the Eppendorf tubes were all empty and tubes and the leaf flaps were removed. Six days later the irrigation of the pots started.

Labelled plants were harvested at 3, 14, 21, 44 and 74 days after labelling (DAL) coinciding with the growth stages V4, V6, R1, R5 and R7 (Fehr et al. 1971). R7 is termed "physiological maturity" but unlike the final R8 stage (harvest maturity) it was found that with care it was possible to recover intact nodules from this sandy soil. The shoot tissue was excised, and all soil was carefully passed through a 2 mm sieve and all visible roots were removed. The sieved soil was thoroughly mixed, dried and weighed and subsampled for subsequent analysis of total N. Shoot material was dried in a forced air oven (65 °C for > 72 h) and subsequently weighed.

For the plants submitted to leaf labelling, roots were separated in the three size categories (cohorts), primary, secondary and fine. Roots were freeze dried and subsequently all "rhizosphere soil" was manually picked from the roots and subsequently mixed together with the bulk soil from the same pot. Clean (nodule-free) roots and nodules were then dried and weighed, and all plant material and sub-samples of soil were finely ground using a roller mill similar to that of Arnold and Schepers (2004).

Subsamples were then analysed for total N content using the semi-micro Kjeldahl procedure as described by Urquiaga et al. (1992). The ^{15}N enrichment of aliquots of soil and plant material, and the ^{15}N abundance of aliquots of the original soil, containing approximately 35 µgN was determined using an automated continuous-flow isotope-ratio mass spectrometer consisting of a Finnigan DeltaPlus mass spectrometer coupled to the output of a Carlo Erba EA 1108 total C and N analyser (Finnigan MAT, Bremen, Germany) in the "John Day Stable Isotope Laboratory" at Embrapa Agrobiologia (Ramos et al. 2001).

The satellite experiment to quantify the contribution of BNF was all harvested at 74 DAL and the shoot tissue was dried, weighed, sub-sampled and the sub-samples ground and analysed for total N and ^{15}N abundance as described for the other plant samples.

Experiment 2: Comparison of stem and leaf-labelling with ^{15}N-enriched urea to determine NdfRh.

For this pot experiment six seeds were planted per pot (Table 1) and nine days later seedlings were removed to leave two plants per pot. The main experiment consisted of a 2×6 factorial design with five replicates. There were two plant-labelling techniques (leaf or stem), and six harvests. The plants were labelled with ^{15}N 39 days after planting. The procedure for leaf labelling was the same as described in

Experiment 1, using 1 ml of a solution of 5 g urea L^{-1} (95.5 atom % ^{15}N) in each Eppendorf tube and the addition amounted to 2.385 mg ^{15}N excess per plant (4.770 mg ^{15}N excess per pot) in both treatments. For the stem labelling a hole (0.5 mm diam.) was drilled into the side of the stem of each plant at approximately 15 mm above the soil surface. A cotton wick was inserted into the hole and the lower end was immersed in 1 ml of the same solution of ^{15}N-enriched urea. Before leaf or stem labelling, the soil surface was protected with aluminium foil and irrigated via a rubber tube as in Experiment 01 and the soil remained protected by the foil until the end of the experiment.

A satellite experiment was established to quantify the contribution of BNF to the soybean as described in Experiment 1. This satellite experiment consisted of 20 pots filled with same soil treated as described in the main experiment, five planted to soybean seeds inoculated with *B. japonicum* strain SEMIA 5080, and five each planted to a non-N_2-fixing reference plant: grain sorghum (cv. BR 310), non-nod soybean (cv. T181) and rice (cv. IAC 4440). All pots, those with soybean destined for leaf labelling and those not labelled, and the non-legume reference plants were randomly allocated to a complete block design with five replicates.

Harvests were made at 3, 12, 26, 46 and 62 days DAL, coinciding with the growth stages V4, V7, R1, R5 and R7 (Fehr et al. 1971). All recovered roots were collected in plastic bags and frozen and then freeze-dried to allow adhering soil to be separated from the roots (McNeill et al. 1997). This adhering "rhizosphere soil" 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 65 °C). Once again it was found possible to recover intact nodules the soil at R7. Roots were freeze dried and rhizosphere soil was separated manually but unlike Experiment 1, analysed for N and ^{15}N enrichment separately, i.e., not added to the bulk soil.

The satellite experiment to quantify the contribution of BNF was all harvested at 62 DAL and the shoot tissue was dried, weighed, sub-sampled and the sub-samples ground and analysed for total N and ^{15}N abundance as described for Experiment 1.

All plant and soil samples were analysed for total N and ^{15}N enrichment/abundance as described for Experiment 1.

Experiment 3: Comparison of different ^{15}N-labelling techniques.

For this experiment three soybean seeds per pot were inoculated with peat-based inoculant consisting of a mixture of two *Bradyrhizobium* strains, SEMIA 5019 and SEMIA 5080 were planted on 22 October 2016 (Table 1). After 14 days the plants were thinned to 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 the mixture of urea and glucose (often labelled with ^{13}C or ^{14}C) has been used in several studies, but the impacts of glucose addition on the distribution of enriched N in the plant or on rhizodeposited N were not studied (e.g. Wichern et al. 2007a; Hupe et al. 2016; Rasmussen et al. 2019).

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 et al. 1971). 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 following the protocol of McNeill et al. (1997) as described for Experiment 1. 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. 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 draining from the plants, the surface of the pot

was covered with aluminium foil and irrigation was achieved by a tube passing through the side of the foil as described for Experiment 1.

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 freeze-dried to allow adhering soil to be separated from the roots. This adhering "rhizosphere soil" was separately dried and weighed as described for Experiment 2. 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 65 °C).

The root systems were divided into primary, secondary and fine roots as before. 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 as described for Experiment 1. The samples of bulk soil and rhizosphere soil were likewise ground using the same type of roller mill.

Samples of all plant material, soil and rhizosphere soil were analysed for total N and ^{15}N enrichment/abundance as described for Experiments 1 and 2.

Calculations

Calculation of total ^{15}N excess (mg)

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

$$\text{mg } ^{15}\text{N} = \left(\text{N}_{\text{total}} \times \text{atom\% } ^{15}\text{N excess}\right)/100 \quad (1)$$

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. Soil from approximately the same area was taken for Experiments 1, 2 and 3, but at different times and the atom % ^{15}N excess of the soil was corrected using the ^{15}N abundance of the soil from unlabelled pots. For the plant material in Experiments 1 and 2, the ^{15}N excess was calculated using the ^{15}N enrichment of atmospheric N (0.3663 atom % ^{15}N—Chalk et al. 2015). For Experiments 1 and 2 the ^{15}N abundance of the total N in the soil was found to be + 4.97 ‰ or 0.00182 atom % ^{15}N excess.

In an effort to have a more accurate evaluation of the natural abundance of unlabelled control plants in Experiment 3, samples were taken from the unlabelled soil and parts of the ten unlabelled plants were analysed and determined to be 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).

Rhizodeposit N according to Janzen and Bruinsma (1989) – root tissue as the source of rhizodeposited N

For the quantification of the rhizodeposited N (all N in the soil not recovered in roots and nodules), the first methodology used was that of Janzen and Bruinsma (1989—subsequently referred to as the "J&B technique") described in the Introduction:

$$\text{Rhizodeposited N} = \text{TNsoil} * \frac{(\text{Atom\% } ^{15}\text{Nexcess soil})}{(\text{Atom\% } ^{15}\text{Nexcess root})}. \quad (2)$$

For all three experiments calculations using the J&B technique were made using roots without nodules and also roots + nodules.

Rhizodeposit N according to Hupe et al (2016) – whole plant as the source of rhizodeposited N

Secondly for the "isotopic mass balance" technique, the calculations followed those of Hupe et al. (2016) but the equations given in that publication and that in

the supplementary information of Hupe et al. (2018) were found to have an error and were thus modified. The correction of these calculations is explained in the Supplementary Information – "Isotopic mass balance technique".

The final equations to calculate N derived from rhizodeposits [NdfRh (mg)] as the sum of the two expressions for ^{15}NdfRh (mg) and ^{14}NdfRh (mg) where:

$$^{15}\text{NdfRh (mg)} = [^{15}\text{N}_{\text{tracer}} \text{ in soil (mg)} * {}^{15}\text{N in plant (mg)}]/[^{15}\text{N}_{\text{tracer}} \text{ in plant (mg)}] \quad (3)$$

and

$$^{14}\text{NdfRh (mg)} = [^{15}\text{N}_{\text{tracer}} \text{ in soil (mg)} * {}^{14}\text{N in plant (mg)}]/[^{15}\text{N}_{\text{tracer}} \text{ in plant (mg)}] \quad (4)$$

For this experiment the whole plant N included that in the shoot, roots and nodules.

This was also expressed in Eq. 4. of Ramussen et al. (2019) as:

$$\%\text{NdfRh} = 100 * \frac{\text{soil } ^{15}\text{N yield}}{\text{soil } ^{15}\text{N yield} + \text{root } ^{15}\text{N yield} + \text{shoot } ^{15}\text{N yield}} \quad (5)$$

where ^{15}N yield is the mass of excess ^{15}N in each compartment (soil, root and shoot).

Calculation of N proportion (%) N derived from BNF (%Ndfa)

For the satellite experiments of Experiments 1 and 2 to quantify the contribution of BNF, the proportion of N in the soybean derived from the air (%Ndfa) was calculated using the equation of Shearer and Kohl (1989):

$$\%\text{Ndfa} = 100 * \left\{\left(\delta\,^{15}\text{N reference plant} - \delta\,^{15}\text{N soybean}\right)/\left(\delta\,^{15}\text{N reference plant} - B\right)\right\} \quad (6)$$

where the value *'B'* is the ^{15}N natural abundance of the N in the soybean derived from the air (BNF). The *'B'* values used for the shoot tissue (*'B_s'*) of the soybeans inoculated with SEMIA 5019 and SEMIA 5080 were -3.86 ‰ and -1.63 ‰, respectively, Guimarães et al. 2008).

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

Experiment 1

In this experiment mean shoot DM increased approximately three-fold from the first sampling at 3 DAL (8 g DM plant^{-1}) until the final harvest at 74 DAL (25 g DM plant^{-1}—Supplementary Information Fig. S1) with no significant difference between the two inoculation treatments. Total mean shoot N accumulation showed a similar increase from 97 to 307 mg N plant^{-1} in the same period (Supplementary Information Figure S2). However, mean total root DM (excluding nodules) increased from 2.3 to 4.0 g DM plant^{-1} in the period from 3 to 21 DAL and then made no further increase up until the final harvest at 74 DAL (3.7 g DM plant^{-1}—Supplementary Information Fig. S3E). Mean root N accumulation showed a similar pattern of increase from 27.2 to 45.2 mg N plant^{-1} from 3 to 21 DAL followed by a gradual decline to 39.4 mg N plant^{-1} at 74 DAL (Fig. 1). The largest proportion of root N was found in the fine roots, constituting 60% of all root N at 74 DAL compared to 17 and 23% for primary and secondary roots, respectively. However, the largest part of the belowground biomass was the nodules. At 3 DAL nodules were being formed and showed a mean mass of 0.25 g plant^{-1} rising to 1.0 g plant^{-1} at 21 DAL (Supplementary Information Fig. S3D). Subsequently the DM of nodules formed by the *B. elkanii* strain, SEMIA 5019 was significantly greater ($P < 0.05$) than that of the *B.*

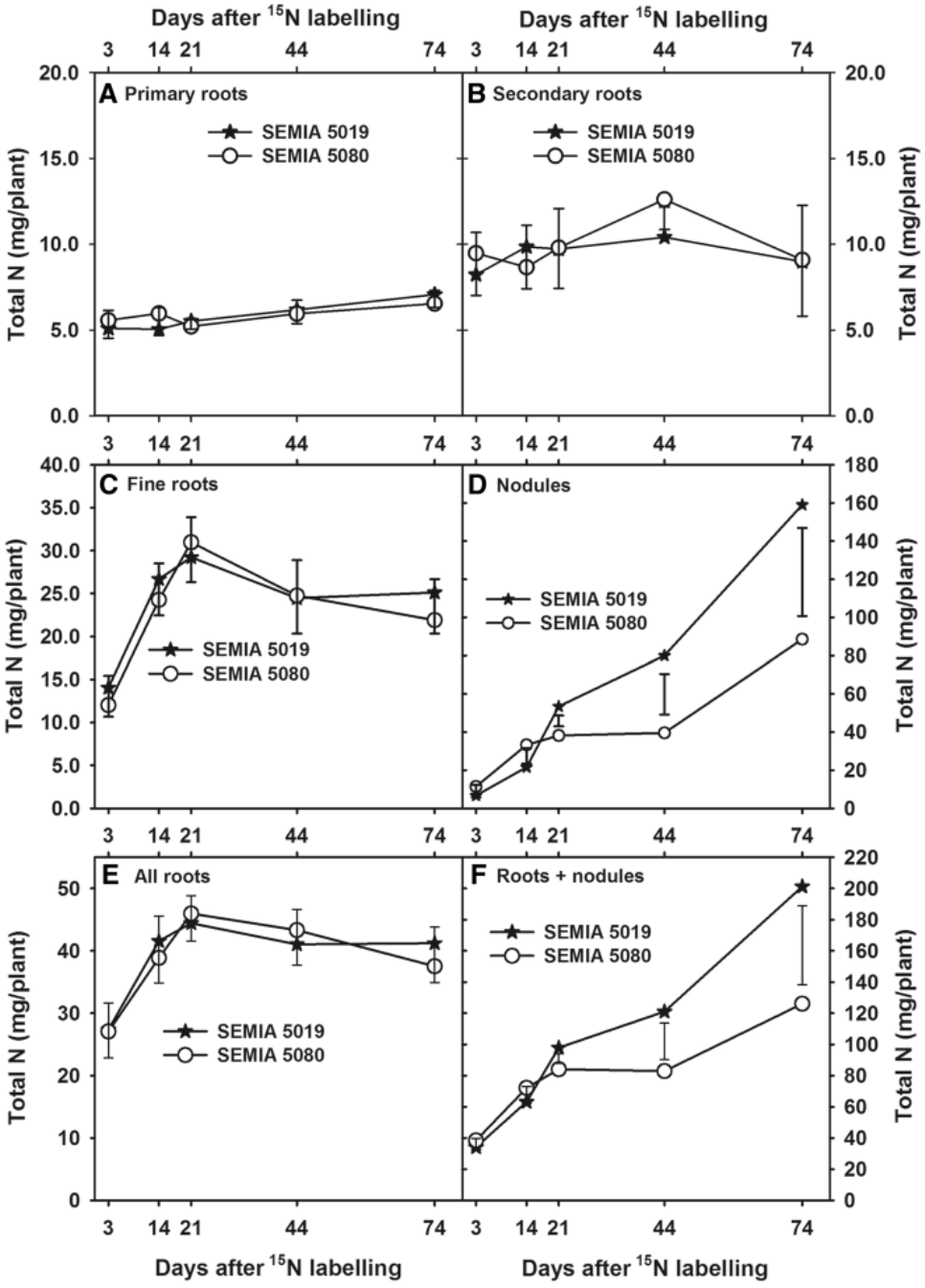

Fig. 1 Total N accumulated by primary, secondary and fine roots and nodules of soybean plants inoculated with *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 from 3 days after ^{15}N labelling (DAL) until final harvest at 74 DAL. Experiment 01. Means are of 4 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

diazoefficiens (SEMIA 5080) at 3.97 and 2.22 g DM plant^{-1}, respectively, at 74 DAL. The total N content of the nodules formed by the SEMIA 5019 and SEMIA 5080 strains was, respectively, 3.7 and 2.4 times that of the clean (nodule-free) roots (Fig. 1).

As was to be expected for plants which tripled their total N content between 3 and 74 DAL, the single addition of ^{15}N enriched N added to the leaves was gradually diluted and the ^{15}N enrichment consequently declined from 1.662 to 0.534 atom % ^{15}N excess (mean of both treatments Supplementary Information Fig. S4). However, the mean ^{15}N enrichment of the roots (without nodules) decreased by only 19% from 0.607 to 0.511 atom % ^{15}N excess from 21 to 74 DAL (Fig. 2E). The ^{15}N enrichment of the nodules was much lower than that of the roots and decreased from 0.453 to 0.283 atom % ^{15}N excess from 21 to 74 DAL (Fig. 2D).

At the final harvest the ^{15}N enrichment of the roots + nodules (nodulated roots) was 0.274 and 0.406 atom % ^{15}N excess for the plants inoculated with the *Bradyrhizobium* strains SEMIA 5019 and SEMIA 5080, respectively (Fig. 2F, Table 2), and that of the soil was, respectively, 0.0287 and 0.0314 atom % ^{15}N excess for the two treatments (Table 2). Three days after labelling approximately 400 µg of excess ^{15}N-enriched N was deposited in the soil amounting to 12% of the total added to the leaves (Fig. 3). Until 21 DAL there was no increase in the excess ^{15}N in the soil and at the final harvest (74 DAL) there was a mean of 668 µg ^{15}N excess deposited in the soil.

Applying the J&B technique for the final harvest using the ^{15}N enrichment of the soil and of the nodulated roots, it was estimated that the total plant N deposited in the soil (NdfRh) was 236.9 and 177.9 mg N plant^{-1}, for the plants inoculated with

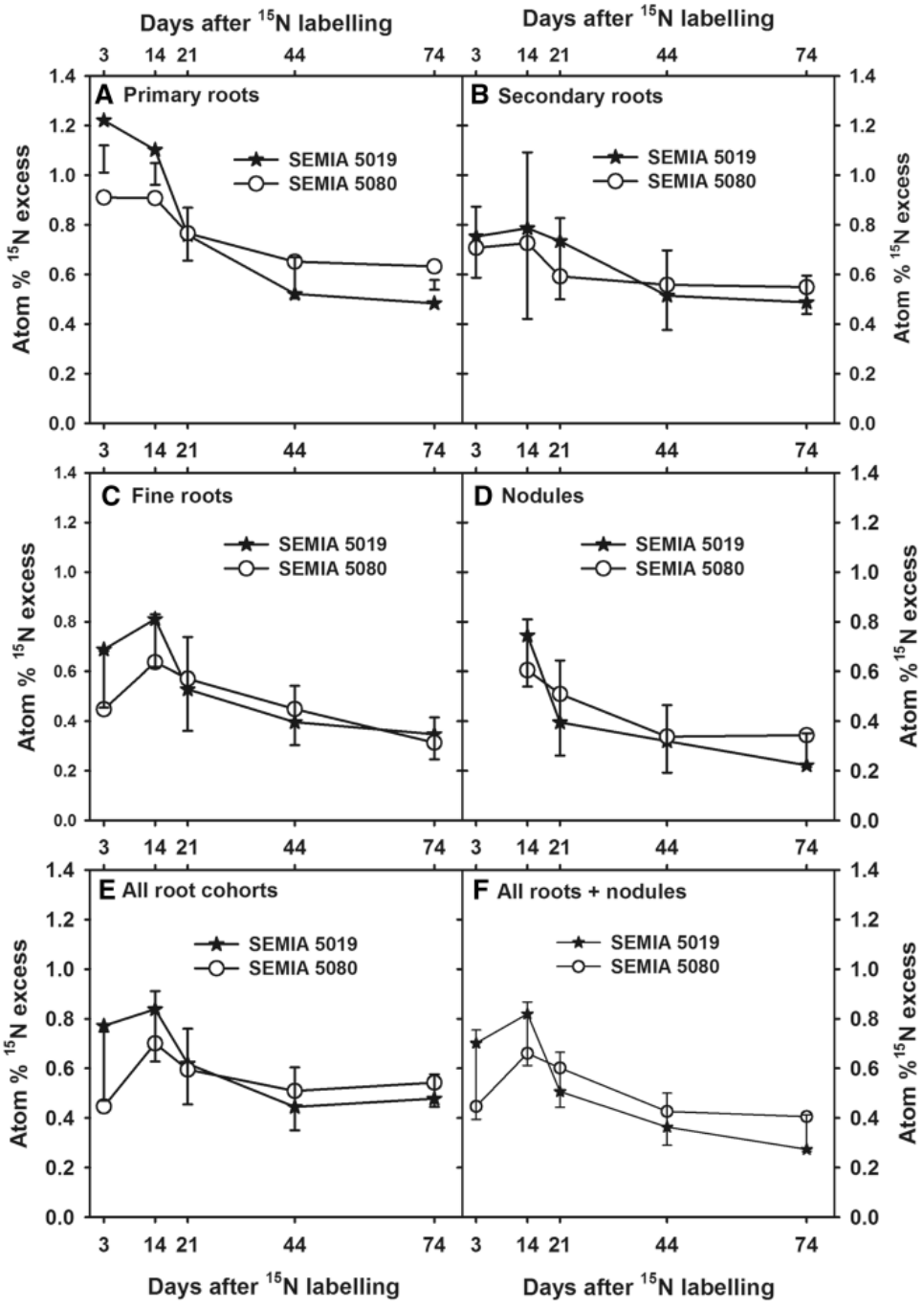

Fig. 2 ^{15}N enrichment (atom % ^{15}N excess) of primary, secondary and fine roots and nodules of soybean plants inoculated with *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 from 3 days after ^{15}N labelling (DAL) until final harvest at 74 DAL. Experiment 01. Means are of 4 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

Table 2 Calculations of N in rhizodeposits calculated from the ^{15}N enrichment of roots or roots plus nodules and the ^{15}N enrichment of soil (after Janzen and Bruisma, 1989) and using the mass balance technique of Hupe et al. (2016) for the final three harvests of soybean in Experiments 2 and 3

Harvest	Treatment	Weighted mean atom % ^{15}N excess				Estimate of N derived from rhizodeposits					
DAL		Soil	Shoot tissue	All roots	All roots + nodules	All roots[a]		All roots + nodules[b]		Mass balance[c]	
	Experiment 02					(mg N)	%	(mg N)	%	(mg N)	
21	*Rhizobium strain 29 W*	0.0212	1.0467	0.6191	0.5068	82.9	23.5	92.4	26.3	50.4	16.0
	Rhizobium strain CPAC7	0.0198	1.0381	0.5954	0.6017	73.4	23.4	71.7	23.4	47.8	16.0
	Mean	0.0205 B[e]	1.0420 A	0.6073 A	0.5542 A	78.2 B	23.4 B	81.9 C	24.8 B	49.1 B	15.9 B
44	*Rhizobium strain 29 W*	0.0205	0.7756	0.4446	0.3633	112.2	28.1	136.2	30.8	64.7	17.8
	Rhizobium strain CPAC7	0.0248	0.9057	0.5094	0.4263	127.2	34.7	149.9	38.5	67.8	22.1
	Mean	0.0227 B	0.8406 AB	0.4770 B	0.3948 B	119.9 A	31.4 A	143.1 B	34.7 A	66.2 B	20.0 A
74	*Rhizobium strain 29 W*	0.0287	0.4547	0.4784	0.2736 b[d]	130.7	19,6	227.1	32.4	125.0	19.0
	Rhizobium strain CPAC7	0.0314	0.6122	0.5427	0.4056 a	1 133.9	24.5	177.9	27.3	104.0	20.3
	Mean	0.0301 A	0.5335 B	0.5105 AB	0.3396 B	133.9 A	22.1 B	207.4 A	29.9 AB	114.5 A	19.7 A
	Coefficient variation (%)	15.3	33.0	21.6	15.9	20.8	25.4	27.9	25.9	21.8	12.5
	Experiment 3					(mg N)	%	(mg N)	%		
26	Leaf labelled	0.0059	2.2621	0.8862	0.8657	57.3	21.4	62.1	22.3	21.1	8.8
	Stem labelled	0.0108	2.2368	1.2009	1.1821	65.5	24.8	67.8	25.2	32.0	13.5
	Mean	0.0084 B	2.2490 A	1.0431 A	1.0238 A	61.4 B	23.1 A	64.9 B	23.8 B	26.6 C	11.1 B
46	Leaf labelled	0.0081	0.7850	0.7383	0.4658	111.0	21.3	177.6	29.5	70.6	14.2
	Stem labelled	0.0108	0.8520	1.0814	0.8173	97.9	17.1	133.2	21.6	80.4	14.6
	Mean	0.0095 AB	0.8185 B	0.9097 A	0.6415 B	1104.5 AB	19.2 AB	155.4 A	25.5 AB	75.5 B	14.4 AB
62	Leaf labelled	0.0129	0.6282	0.8330	0.4949	139.3	22.8	190.3	28.7	118.3	19.9
	Stem labelled	0.0144	0.4763	0.8939	0.4384	121.9	13.4	174.8	18.1	135.1	13.7
	Mean	0.0137 A	0.5523 B	0.8635 B	0.4667 C	130.6 A	18.1 B	182.6 A	23.4 A	126.7 A	16.8 A
	Coefficient variation (%)	40.1	35.4	24.2	28.5	44.5	36.3	37.0	30.2	42.2	35.5

[a]After Janzen and Bruinsma (1989) using weighted mean ^{15}N enrichment of roots without nodules as the source of rhizodeposits

[b]After Janzen and Bruinsma (1989) using weighted mean ^{15}N enrichment of roots and nodules as the source of rhizodeposits

[c]Mass balance technique as applied by Hupe et al (2016; 2018) but modified as described in the Materials and methods

[d]Means in the same column for the same harvest followed by the same lower-case letter are not at significantly different at $P < 0.05$ (Student LSD test)

[e]Means in the same column followed by the same uppercase letter are not at significantly different at $P < 0.05$ (Student LSD test)

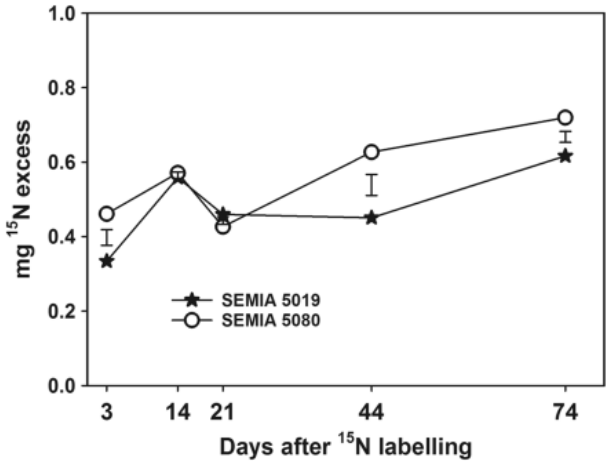

Fig. 3 ^{15}N tracer (mg ^{15}N excess) recovered in soil from soybean plants inoculated with *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 from 3 days after ^{15}N labelling (DAL) until final harvest at 74 DAL. Experiment 01. Means are of 4 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

SEMIA 5019 and SEMIA 5080 respectively, amounting to 32 and 27% of all plant N (Table 2). The estimates of NdfRh using the "mass balance" technique of Hupe et al. (2016) for data from the same harvest were 125.0 and 104.0 mg plant^{-1} for the plants inoculated with SEMIA 5019 and SEMIA 5080 respectively, amounting to 19 and 20% of all plant N.

Estimates of the %NdfRh when using clean roots, nodulated roots or nodules only as the source of NdfRh for the J&B technique, when ^{15}N tracer deposited in the soil until 3 DAL was either discounted or not, are given in Table 5.

In the satellite experiment to quantify the contribution of BNF to the shoot tissue of the soybean, the ^{15}N abundance of the three non-N_2-fixing reference plants were + 13.78 + 15.12, + 13.63 ‰ for the rice, sorghum and spiderwort, respectively. The shoot tissues of the soybean plants inoculated with *Bradyrhizobium* strains SEMIA 5019 and SEMIA 5080, were + 2.12 and + 1.91 ‰. The proportion of shoot N derived from BNF was calculated (Eq. 06) using 'B' values of -3.86 and -1.63 ‰ for the strains SEMIA 5019 and SEMIA 5080 respectively (Guimarães et al. 2008). The proportion of plant N derived from BNF were estimated to be 66.8 ± 1.5 and 77.6 ± 1.1%, for the plants inoculated with SEMIA 5019 and SEMIA 5080, respectively.

Experiment 2

From the first sampling at 3 DAL until the final harvest at 62 DAL, shoot DM increased from a mean of 2.3 to approximately 20 g DM plant^{-1} (Supplementary Information Fig. S5). The DM of roots without nodules decreased from a mean of 1.5 g to 1.1 g plant^{-1} from the first sampling at 3 DAL to the third sampling at 26 DAL and subsequently increased to 1.5 g plant^{-1} at the final sampling at 62 DAL (Supplementary Information Fig. S6). The accumulation of DM and N by the nodules of the stem-labelled plants was significantly higher than that of the leaf-labelled plants at the last harvest (Supplementary Information Fig. S6D, Fig. 4D). Total N in accumulated in roots decreased from a mean of 22.4 mg at 3 DAL to 14.7 mg at 12 DAL and attained a mean of 18.4 mg N pot^{-1} at the final harvest at 62 DAL (Fig. 4E). As was the case in Experiment 01, the fine roots had the greatest DM and N content with 51% of all root N (without nodules) at 62 DAL (Fig. 4). However, total N accumulated in the nodules of the leaf-labelled plants reached a maximum of 19.3 mg pot^{-1} at 46 DAL and a maximum of 34.9 mg pot^{-1} at 62 DAL in the stem-labelled plants, 1.7 and 2.3 times that in the roots without nodules at the same sampling dates, respectively (Fig. 4D, E). In the period from 46 to 62 DAL total N accumulation of the roots + nodules decreased from a mean of 32 mg N plant^{-1} to 29 mg plant^{-1} for the leaf-labelled plants and increased to 50 mg N pot^{-1} for the stem-labelled plants (Fig. 4). This suggested that the stem labelling stimulated nodule growth and N_2 fixation and this was reflected in the high shoot N accumulation at this time.

As was expected, the ^{15}N enrichment of the shoot tissue decreased steadily from a mean of 4.23 atom % ^{15}N excess at 3DAL to 0.552 atom % ^{15}N at 62 DAL (Supplementary Information Fig. S7). After an initial rise at 3 DAL the ^{15}N enrichment of the roots showed a slow decline until the final harvest at 62 DAL (Fig. 5). Owing to the increase in the accumulation of N of low ^{15}N enrichment by the nodules at the later stages of growth (Fig. 5D), the weighted mean ^{15}N excess of the roots plus nodules showed a considerable decline from approximately 1.01 atom % ^{15}N excess at 24 DAL to 0.545 atom %^{15}N excess at the final harvest at 62 DAL (Fig. 5F).

At the final harvest the ^{15}N enrichment of the roots + nodules (nodulated roots) was 0.495 and 0.438

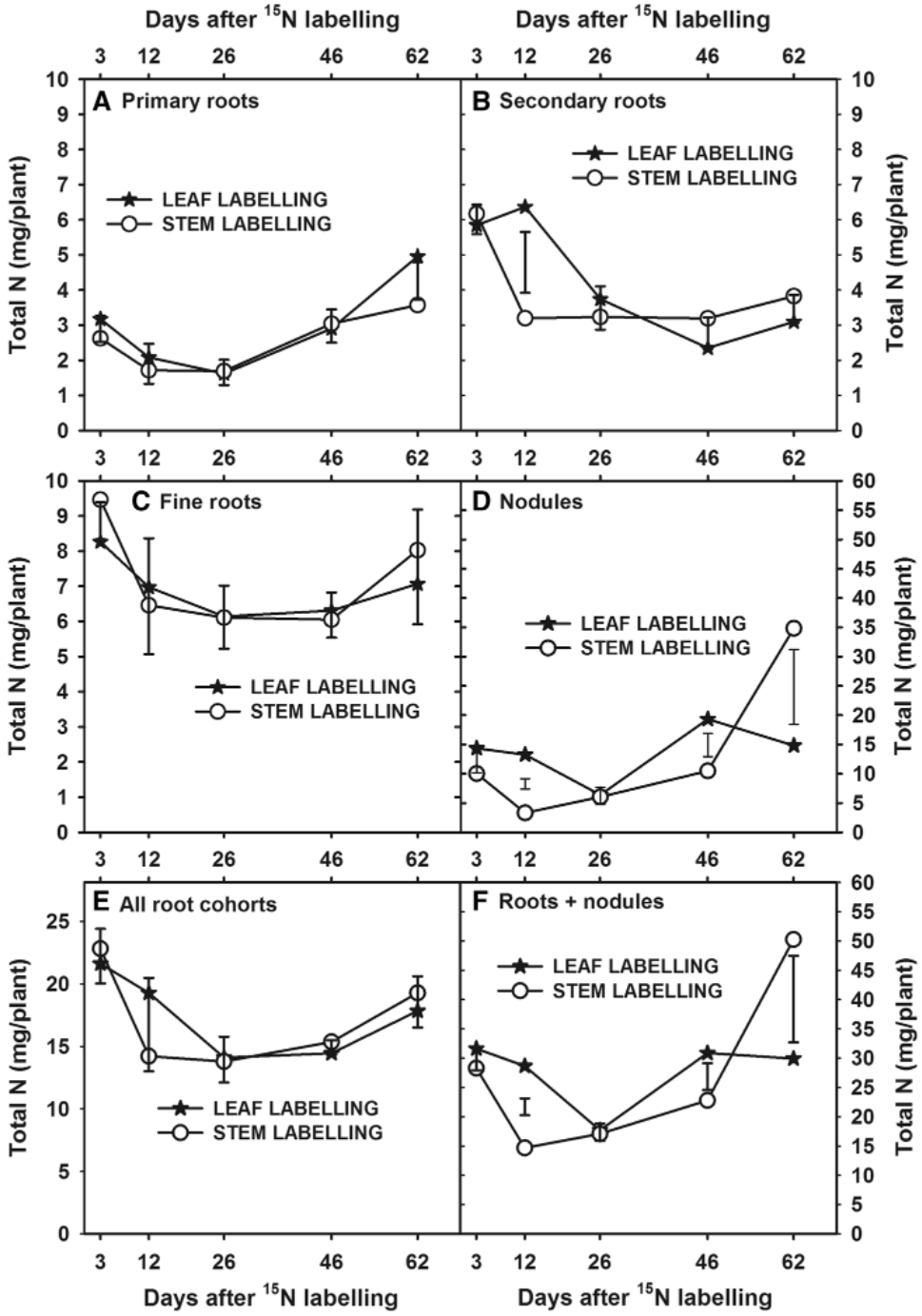

Fig. 4 Total N accumulated by primary, secondary and fine roots and nodules of soybean plants inoculated with *Bradyrhizobium* strain SEMIA 5080 and either leaf labelled or stem labelled with ^{15}N enriched urea, from 3 days after ^{15}N labelling (DAL) until final harvest at 62 DAL. Experiment 02. Means are of 5 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

atom % ^{15}N excess for the leaf-labelled and stem-labelled plants, respectively (Table 2), and that of the soil was 0.0129 and 0.0114 atom % ^{15}N excess, respectively, for the two treatments.

Three days after labelling, approximately 500 µg of excess ^{15}N-enriched N was deposited in the soil amounting to 21% of the total added to the leaves (Fig. 6). Until 12 DAL there was no increase in the excess ^{15}N in the soil and in the case of the leaf labelled plants there was no increase in the ^{15}N excess in the soil until 26 DAL. At the final harvest (62 DAL) there was a mean of 1030 µg ^{15}N excess deposited in the soil.

Applying the J&B technique for the final harvest at 62 DAL using the ^{15}N enrichment of the soil and the nodulated roots, it was estimated that the total plant N deposited in the soil was 195 and 200 mg N plant^{-1}, for the leaf-labelled and stem-labelled plants,

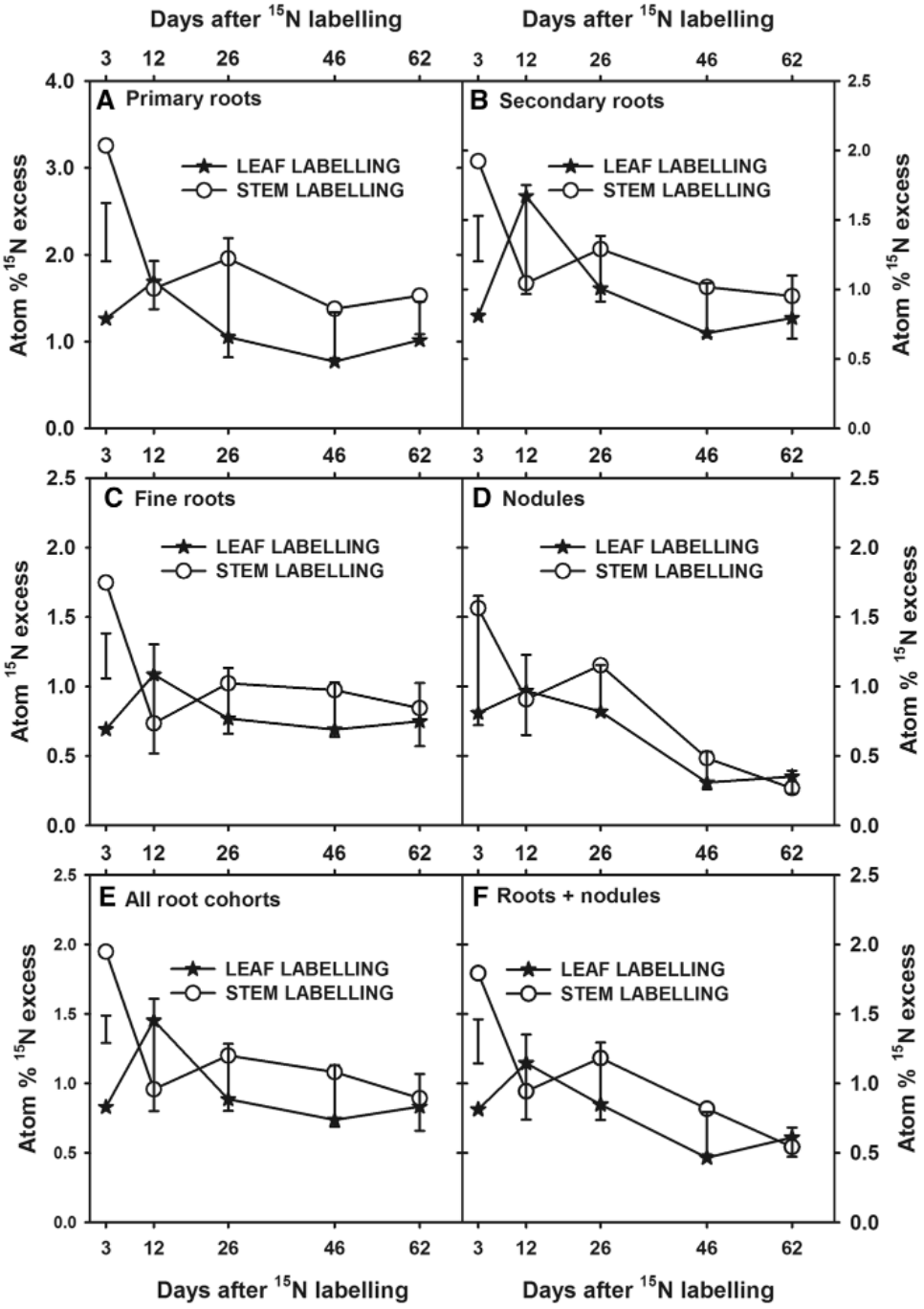

Fig. 5 ^{15}N enrichment (atom % ^{15}N excess) of primary, secondary and fine roots and nodules of soybean plants inoculated with *Bradyrhizobium* strain SEMIA 5080 and either leaf labelled or stem labelled with ^{15}N enriched urea from 3 days after ^{15}N labelling (DAL) until final harvest at 62 DAL. Experiment 02. Means are of 5 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

respectively, amounting to 30 and 21% of all plant N (Table 2). The estimates using the "mass balance" technique of Hupe et al. (2016) for data from the same harvest were 117 and 134 mg plant^{-1} for the leaf-labelled and stem-labelled plants, respectively, amounting to 20 and 14% of all plant N.

Estimates of the %NdfRh when using clean roots, nodulated roots or nodules alone as the source of NdfRh for the J&B technique, when ^{15}N tracer deposited in the soil until 3 DAL was either discounted or not, are given in Table 5.

The mean ^{15}N abundance of the three non-N_2-fixing control crops, were respectively (means ± standard errors) + 10.2 ± 0.3, + 11.4 ± 0.5 and + 11.0 ± 0.3 ‰ for the sorghum, rice and non-nod soybean, and the soybean inoculated with a *Bradyrhizobium* strain SEMIA 5080 (CPAC 7) was + 0.52 ± 0 4 ‰. The proportion of plant N derived from BNF was calculated

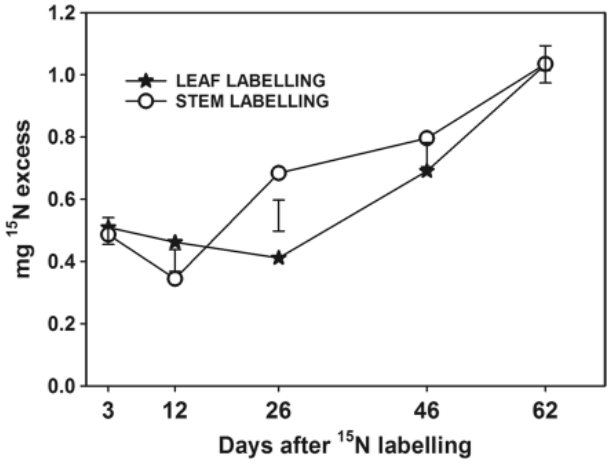

Fig. 6 ^{15}N tracer (mg ^{15}N excess) recovered in soil from soybean plants inoculated with *Bradyrhizobium* strain SEMIA 5080 and either leaf labelled or stem labelled with ^{15}N enriched urea from 3 days after ^{15}N labelling (DAL) until final harvest at 74 DAL. Experiment 02. Means are of 5 replicates. Error bars indicate Least Significant Differences between means (LSD t-test)

as described in the Materials and methods using the equation (Eq. 06) of Shearer and Kohl (1986) and the 'B' value of -1.63 ‰ for the strain CPAC 7 (Guimarães et al. 2008). The proportion of plant N derived from BNF was estimated to be 82 ± 1.2%.

Experiment 3

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 NdfRh at the final harvest.

There were no differences in DM or N accumulation of the soybean plants between the treatments with the four different leaf-labelling strategies (Supplementary Information Fig. S8 and Fig. 7). 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 DAL) essentially ceased. Nodule and root DM continued to increase until 47 DAL and then significantly decreased until final harvest (Supplementary Information Fig. S8). 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 (Fig. 7). This continued increase in N accumulation during the grain filling stage V5 to V6 (14 to 47 DAL) when nodule DM and N content were also increasing, indicated that N_2 fixation was still active. The final stage for R5 to R7 (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% (Supplementary Information Fig. S8C and Fig. 7). Between 7 and 10 DAL, shoot N accumulation ceased, but subsequently N accumulation continued (Fig. 7A). This interruption of N accumulation may be attributed to the trauma induced by the leaf cutting and/or 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 (Fig. 7). In the last 23 days the N lost by the nodules amounted to 51 mg plant^{-1} (Fig. 7D).

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 2385 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. 8). 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. 9). 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 (Fig. 9A, B). 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

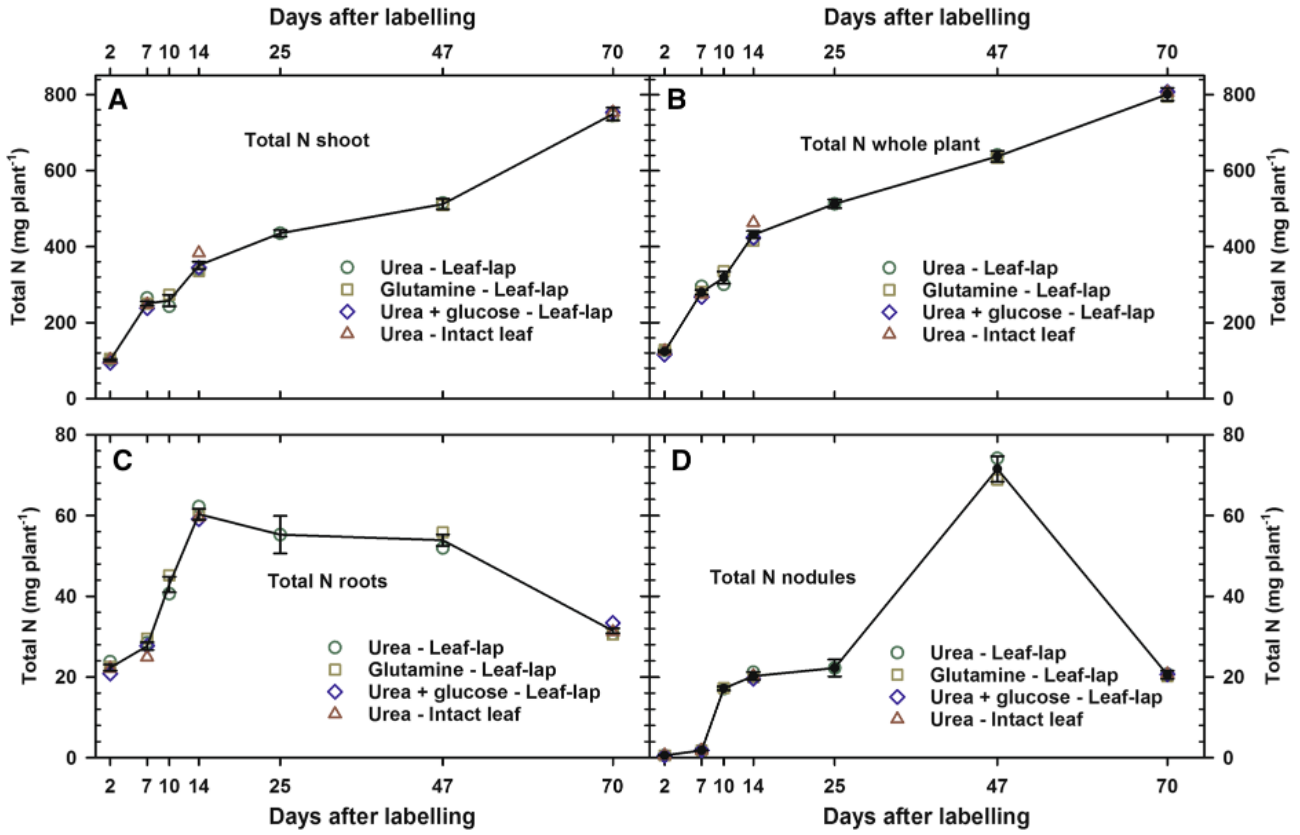

Fig. 7 Total N accumulated by roots, shoots and nodules of soybean plants inoculated with a mixture of *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 and leaf labelled with ^{15}N-enriched urea, glutamine or urea + glucose using the leaf-flap technique of Khan et al (2002a) or intact leaves labelled with ^{15}N-enriched urea. Data are for harvests from 2 to 70 days after labelling and means of 5 replicates. Experiment 3. Error bars indicate Least Significant Differences between means (LSD t-test)

Fig. 9D). As the plants leaf-labelled with urea (the three treatments ULL, USLL and UEL) 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 3).

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 tracer 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 on DM or N accumulation by the shoot or root tissues (Supplementary Fig. S08, Fig. 7).

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

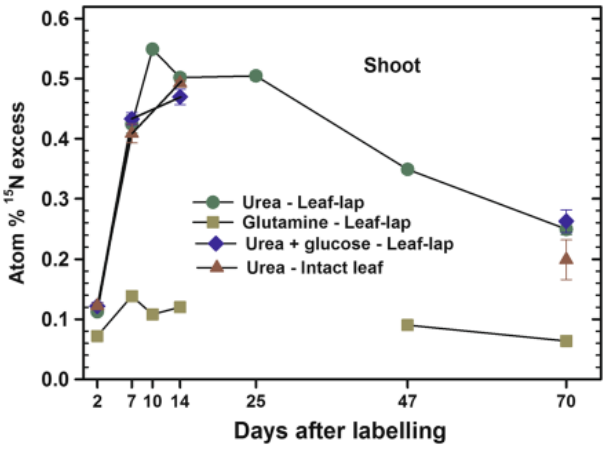

Fig. 8 ^{15}N enrichment (atom % ^{15}N excess) of shoots of soybean plants inoculated with a mixture of *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 and leaf labelled with ^{15}N-enriched urea, glutamine or urea + glucose using the leaf-flap technique of Khan et al (2002a) or intact leaves labelled with ^{15}N-enriched urea. Data are for harvests from 2 to 70 days after labelling and means of 5 replicates. Experiment 3. Error bars indicate Least Significant Differences between means (LSD t-test)

tracer in the shoot and roots + nodules, respectively, at 2 DAL (Table 3). After 10 DAL the % recovery of applied ^{15}N tracer 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 tracer (11.7%) peaked at 14 DAL, and for the glutamine labelled treatment reached 14.3% of applied tracer at the final harvest.

Applying the J&B technique to the urea-labelled treatments using the ^{15}N enrichment of the soil (including rhizosphere soil) and of the nodulated roots it was estimated that between 71 and 107 mg of plant N were deposited in the soil, between 8 and 12% of all plant N (Table 4). Using the mass balance technique of Hupe et al (2016) these estimates were somewhat lower (differences not significant at $P < 0.05$) ranging from 56 to 89 mg of plant N deposited in the soil equivalent to between 7 and 10% of total plant N. However, the results 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.

Estimates of the %NdfRh when using clean roots, nodulated roots or nodules alone as the source of NdfRh for the J&B technique, when ^{15}N tracer deposited in the soil until 2 DAL was either discounted or not, are given in Table 5.

Discussion

Differences in the results between the three experiments

The same soybean variety (cv. Celeste) was used for the first two experiments. The inoculation with the rhizobium strains produced abundant nodulation ranging from 0.7 to 2.5 g DM plant^{-1} for the plants inoculated with the rhizobium strain SEMIA 5080 and 4.0 g DM plant^{-1} for those plants in Experiment 1 inoculated with strain SEMIA 5019 (Supplementary Information Figs S 03 and S 06). The greater DM of nodules formed with the strain SEMIA 5019 has been observed in many earlier studies (see Discussion in Pauferro et al. 2010). The total N accumulation of the plants was between 251 and 531 mg N plant^{-1}. The estimates of the %NdfRh at the final harvest using the J&B technique discounting the initial leakage of tracer N using the ^{15}N enrichment of clean roots ranged from 9 to 11% for Experiment 1 and 6 to 12% for Experiment 2 (Table 5). Using the ^{15}N enrichment of nodulated roots the estimate ranged from 12 to 18% and 8 and 15% for Experiments 1 and 2, respectively.

The soybean variety used in Experiment 3 was BRS 360RR and inoculation with rhizobium resulted in a mean nodule mass for the four treatments of 1.5 g DM plant^{-1} (Supplementary Information Fig S 08). Total N accumulation (root + shoot) was very similar for all treatments ranging from 795 to 807 mg plant^{-1}, considerably greater than the plants in Experiments 1 and 2. The different variety of soybean and the greater N accumulation may explain the why the estimates of %NdfRh at the final harvest were considerably different to those for the other two experiments. The estimates of the %NdfRh using the J&B technique with the ^{15}N enrichment of clean roots ranged from 1.5 to 5.5% for the different labelling treatments. Using the ^{15}N enrichment of nodulated roots the estimates ranged from 2.0 to 7.1%.

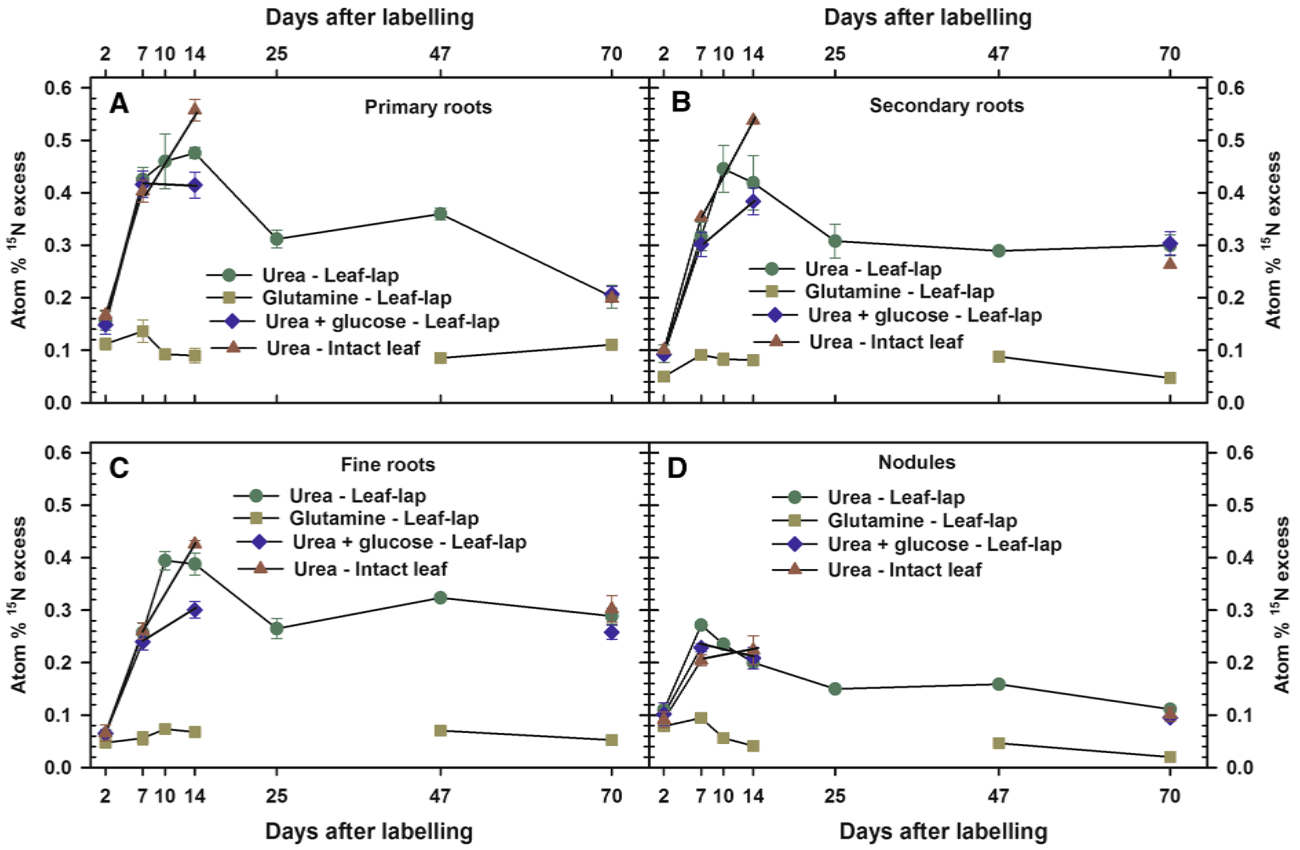

Fig. 9 ^{15}N enrichment (atom % ^{15}N excess) of primary, secondary and fine roots and nodules of soybean plants inoculated with a mixture of *Bradyrhizobium* strains SEMIA 5019 or SEMIA 5080 and leaf labelled with ^{15}N-enriched urea, glutamine or urea + glucose using the leaf-flap technique of Khan et al (2002a) or intact leaves labelled with ^{15}N-enriched urea. Data are for harvests from 2 to 70 days after labelling and means of 5 replicates. Experiment 3. Error bars indicate Least Significant Differences between means (LSD t-test)

Changes in distribution of tracer N over time

The results of Experiment 1, 2 and 3 all showed a strong decline in ^{15}N enrichment of shoot tissue with time after leaf labelling (Supplementary Information Fig. S3, S7 and Fig. 8) although in Experiment 3 this decline was only apparent after 10 DAL. In all other studies where the change in ^{15}N enrichment of shoot tissue with time was studied, the same decline with time was recorded (McNeill et al. 1997, 1998; McNeill and Fillery 2008; Gasser et al. 2015; Hupe et al. 2016; Rasmussen et al. 2019). This is to be expected, owing to the dilution of the fixed amount of added enriched N with increasing unlabelled N derived from soil and/or BNF. However, in our studies after approximately 21 to 26 DAL, ^{15}N enrichment of roots (all root cohorts without nodules) remained approximately stable only showing a mean decline from this time until the final harvest of 16, 17, and 19% in the experiments 1, 2 and 3, respectively (Figs. 2, 5 and 9). This was largely due to the stability of ^{15}N enrichment of the fine roots which in this period held between 49 and 67% of all N in clean roots (i.e. not including nodules) (Supplementary Information Figs S3, S6 and S8). The aforementioned studies of the temporal changes in ^{15}N enrichment of shoots and roots of labelled legumes also found that after periods of seven to ten days, ^{15}N enrichment of the roots tends to stabilise.

With regard to the deposition of ^{15}N-enriched N into soil, it was found that after only three days (Experiments 1 and 2) or two days (Experiment 3) after leaf labelling there was a considerable quantity of enriched N in the soil. Both Gasser et al. (2015) and Rasmussen et al. (2019) obtained similar results, finding significant ^{15}N-enriched N in the

Table 3 Recovery of applied ^{15}N excess (mg) in the soybean shoots, roots, soil and rhizosphere soil as a percentage of that applied as ^{15}N- labelled urea or labelled glutamine. Experiment 3

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
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

soil after only 24 h of labelling leaves of red (*Trifolium pratense*) or white clover (*T. repens*). This was also reported by Gardner et al (2012) for sub-clover (*T. subterraneum*) and lucerne (*Medicago sativa*). Another observation common to our results and these studies was that the increase in the deposition of enriched N was gradual after the first two or three days in our study, or after one day in the studies of Gardner et al. (2012), Gasser et al. (2015) and Ramussen et al. (2019). In our three experiments the quantity of ^{15}N-enriched N found in the soil at final harvest of the soybean (between 62 and 74 DAL) was only approximately twice that of the quantity registered two or three DAL. Gardner et al. (2012) found no significant increase in ^{15}N enrichment of the soil after one day until the final harvest 32 DAL. It thus seems that the pattern of deposition of excess ^{15}N in the soil follows an initial (one to three days) rapid increase followed by only gradual increase over much longer periods. This pattern was essentially the same when labelled urea was substituted by ^{15}N labelled glutamine.

In our Experiment 3 on soybean in the treatments leaf-labelled with urea, by 2 DAL approximately 5% of the ^{15}N tracer 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

Table 4 Calculations of N in rhizodeposits calculated from the ^{15}N enrichment of roots or roots plus nodules and the ^{15}N enrichment of soil (after Janzen and Bruisma, 1989) and using the mass balance technique of Hupe et al. (2016) for three harvests of soybean in Experiment 4

Harvest	Treatment	Weighted mean atom % ^{15}N excess				Estimate of N derived from rhizodeposits					
DAL		Soil + rhizosphere soil	Shoot tissue	All roots	All roots + nodules	All roots[a]		All roots + nodules[b]		Mass balance[c]	
						(mg N)	%	(mg N)	%	(mg N)	%
14	Leaf-lap Ureia (ULL)	0.00153	0.4843	0.4213	0.3652	34.3 cd	7.6 b	39.3 b	8.6 b	30.8 b	6.9 b
	Leaf-lap Gln (GLL)	0.00061	0.1192	0.0766	0.0682	78.7 a	16.2 a	88.9 a	17.8 a	53.1 a	11.6 a
14	Leaf-lap Ureia + G (USLL)	0.00159	0.4697	0.3448	0.3109	42.8 bc	9.4 b	47.6 b	10.3 b	33.6 b	7.5b
	Entire leaf Urea (UEL)	0.00281	0.4778	0.4869	0.4218	55.2 b	11.3 b	63.7 b	12.8 b	57.6 a	11.7 a
	Coefficient variation (%)					28.2	28.2	29.7	29.0	21.3	24.2
47	Leaf-lap Ureia (ULL)	0.00219	0.3488	0.3251	0.2280	57.7 a	8.3 a	82.4 a	11.5 a	57.9 a	8.4 a
	Leaf-lap Gln (GLL)	0.00075	0.0705	0.0772	0.0599	78.0 a	10.9 a	103.1 a	13.8 a	100.1 a	13.4 a
	Coefficient variation (%)					22.2	20.1	29.3	24.1	43.5	42.0
70	Leaf-lap Ureia (ULL)	0.00219	0.2495	0.2650	0,2029	82.6 ab	9.6 ab	107.2 ab	12.1 ab	88.6 b	10.2ab
	Leaf-lap Gln (GLL)	0.00076	0.0525	0.0711	0.0509	100.8 a	11.2 a	140.9 a	15.0 a	149.5 a	15.5 a
70	Leaf-lap Ureia + G (GLL)	0.00173	0.2627	0.2492	0.1911	63.0 bc	7.2 bc	81.4 b	9.1 b	62.1 b	7.1 b
	Entire leaf Urea (UEL)	0.00147	0.2437	0,2551	0.1927	53.3 c	6.2 c	70.8 b	8.2 b	56.4 b	6.6 b
	Coefficient variation (%)					24.0	25.5	26.6	26.6	49.4	41.2

[a]After Janzen and Bruinsma (1989) using weighted mean ^{15}N enrichment of roots without nodules as the source of rhizodeposits

[b]After Janzen and Bruinsma (1989) using weighted mean ^{15}N enrichment of roots and nodules as the source of rhizodeposits

[c]Mass balance technique as applied by Hupe et al (2016; 2018) but modified as described in the Materials and methods

[d]Means in the same column for the same harvest followed by the same letter are not at significantly different at $P < 0.05$ (Student LSD test)

Table 5 Estimates of NdfRh (mg plant^{-1}) and %NdfRh[a] at the final harvest (physiological maturity R7) using the Janzen and Bruinsma (1989) methodology and assuming that the source of rhizodeposited N was either roots without nodules, roots with nodules or solely nodules. The estimates were based on the measured ^{15}N enrichment of these sources, or the enrichments adjusted by discounting the ^{15}N tracer deposited in the soil in the first three (Experiments 1 and 2) or two (Experiment 3) DAL

Experiment	Estimates of rhizodeposited N											
	Treatment	Not discounting initial ^{15}N tracer leakage						Discounting initial ^{15}N tracer leakage					
	Source deposited N (^{15}N enrichment)	All roots without nodules		Roots + nodules weighted mean		Nodules only		All roots without nodules		Roots + nodules weighted mean		Nodules only	
		NdfRh (mg)	%NdfRh	NdfRh (mg)	%NdfRh	NdfRh (mg)	%NdfRh	NdfRh (mg)	%NdfRh	NdfRh (mg)	%NdfRh	NdfRh (mg)	%NdfRh
[b]01	*Bradyrhizobium* SEMIA 5019	130	19.6	227	32.4	279	37.1	59	11.1	103	18.0	128	21.2
	Bradyrhizobium SEMIA 5080	133	24.5	178	27.3	210	30.8	47	9.2	64	11.9	76	13.8
[c]02	Leaf lap labelled	70	22.8	95	28.7	570	70.7	32	11.8	43	15.4	237	52.2
	Stem labelled	61	13.4	88	18.1	338	46.0	24	5.6	34	7.9	130	24.7
[c]03	Leaf lap (urea)	81	9.2	105	11.7	192	19.4	46	5.5	61	7.1	111	12.2
	Leaf lab (glutamine)	100	11.1	139	14.9	353	30.7	18	2.2	25	3.1	64	7.5
	Leaf lap (urea + glucose)	63	7.2	82	9.2	163	16.8	15	1.8	20	2.4	39	4.6
	Entire leaf (urea)	52	6.1	69	7.9	131	14.0	13	1.5	17	2.0	32	3.8

[a]The % of plant N deposited in the rhizosphere expressed as a fraction of total N recovered in the plant + the estimate of the total NdfR

[b]Values are means of four replicates

[c]Values are means of five replicates

labelled N, thus amounting to almost half of the N exported from the labelled leaf to the shoot, roots and soil (Table 3). There was a similar behaviour of the excess ^{15}N deposited in the soil derived from labelled glutamine. At 2 DAL, 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. In such a short space of time it is inconceivable that roots or nodules of undisturbed unlabelled plants would suddenly contribute such significant amounts of rhizodeposits.

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 for the short-term ^{15}N leakage. In Experiment 3 two for the treatments were labelled with enriched urea (ULL and USLL) the leaves were cut (leaf-flap technique – Khan et al. 2002b) 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.

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. We agree with the proposal of Gasser et al (2015) that this initial release of labelled N should be discounted from the total rhizodeposited N. In the case of Gasser et al (2015) discounting of this early leakage of enriched N reduced the estimate of NdfRh by 41% (equivalent to a 70% overestimate of uncorrected value).

In this present study correcting for initial leakage (until 3 DAL) the estimates of NdfRh using the J&B technique and ^{15}N enrichment of nodulated roots decreased the mean estimate of NdfRh from 207 to 84 mg N plant^{-1} (%NdfRh from 30 to 15%) in Experiment 1 and from 183 to 77 mg N plant^{-1} (%NdfRh from 23 to 12%) in Experiment 2 (Table 5). For Experiment 3 by discounting the ^{15}N tracer present in the soils after 2 days the estimates of NdfRh using the J&B technique with nodulated roots were reduced from 105, 139, 82 and 70 to 61, 25, 20 and 17 mg N plant^{-1} for the treatments ULL, GLL, USLL and UEL, respectively. The reductions in the estimates in this experiment ranged from 41 to 76% and on average reduced %NdfRh from 11 to 4% (Table 5).

Comparison of the Janzen and Bruinsma (1989) the mass balance technique (Hupe et al. 2016).

As mentioned in the Introduction, the J&B technique assumes that the roots are the source of rhizodeposits and the calculation of the rhizodeposited N (NdfRh) is based on the dilution of the ^{15}N enrichment of root-derived N by soil N. For this calculation to give an accurate estimate of NdfRh it is assumed that the ^{15}N enrichment of the roots is uniform in space and time. Our results indicate that after 10 DAL the roots, especially the fine roots where senescence and decomposition is likely to be most intense, do not suffer large changes of ^{15}N enrichment with time. This seems to be approximately true for all the studies where changes in root ^{15}N enrichment with time were reported (McNeill et al. 1997; 1998; McNeill and Fillery 2008; Gasser et al. 2015; Hupe et al. 2016; Rasmussen et al. 2019).

The greatest difference between the J&B technique and the mass balance approach is that for the mass balance technique the source of rhizodeposited N is considered to be the whole plant and not just the roots. Typically, at late harvests root N is only a small fraction of the total plant N, especially in the case of soybean. For our three experiments on soybean, at the final harvest the ratios of total shoot N to total root N (without nodules) were 7.8 to 19.0 and 18.4 for the experiments 1, 2 and 3 respectively. Unlike roots, in all relevant studies the ^{15}N enrichment of the shoots declined with time. Furthermore, in this study and those of other authors, aboveground tissues were more highly enriched in ^{15}N than roots. Therefore,

inherent in the mass balance technique as applied by Hupe et al. (2016, 2018, 2019) and Rasmussen et al (2019) is the supposition that throughout the post-labelling stage until harvest the largest proportion of the of rhizodeposited N was derived from the shoot tissues, and only a small proportion from the roots. In this case the roots were acting a conduit for most N that was transferred from the shoot to the soil, and as was the case for the initial deposition of highly labelled N, this N must have been in solution.

As the mass of ^{15}N-enriched N recovered in the soil (the rhizodeposited N) is in the numerator of the equation and that of the whole plant + rhizodeposits are in the denominator (Eq. 05) the estimate of the proportion of rhizodeposited N is always lower than that of the J&B technique (Tables 1 and 2). Unless it is assumed that rhizodeposited N is more highly enriched with ^{15}N than roots, the mass balance technique will always underestimate NdfRh.

Impact of nodulation on estimates of rhizodeposited N

Total nodule DM and N of legumes in studies on rhizodeposition by other authors have rarely been reported. The work of Russell and Fillery (1996a; 1996b) on lupins addressed the problem of the proportion of belowground biomass (BGB) N in the nodules. These authors reported that 33% of BGB was nodule DM and 50% of the N. In our studies this proportion was 46, 38 and 31% of BGB-DM and 76, 62 and 59 of BGB-N for experiments 2, 3 and 4, respectively. There seems to be no other reports of the proportions of DM and N in the recovered roots, but several authors have mentioned that the low ^{15}N enrichment of nodules will lead the non-uniform distribution of ^{15}N enrichment in the roots (McNeill et al. 1997; McNeill and Fillery 2008). Khan et al. (2002a) wrote "the enrichment of recovered roots to be applied to the soil fraction, the ratio of nodule:root material in the soil fraction would need to be identical to the ratio of nodules:roots of recovered roots". They suggested that this requirement may seldom be met. In our experiments with soybean the contribution of nodule N to recoverable belowground plant N was 76, 62 and 40% for Experiments 1, 2 and 3 respectively. Between growth stages R7 and R8, nodules are observed to senesce and it seems logical to assume that most of this N remains in the soil. However, if the ^{15}N enrichment of nodules is used as a source of N in the J&B technique and initial leakage of tracer N is discounted the contribution of NdfRh ranges from 14 to 71% of whole plant N and in all cases this is considerably above the total N in nodules at the final two harvests (Table 5, Figs. 1, 4 and 7).

Our results show that using J&B technique the ^{15}N enrichment of nodulated roots (weighted mean roots + nodules) gave on average for the three final harvests of Experiments 1, 2 and 3, respectively, estimates of NdfRh 30, 54 and 31% higher than when nodule-free roots were utilised. Using only the data from the final harvest of each experiment these differences were greater by 81, 71 and 30% (Table 5).

The nodules at final harvest are senescent and difficult to sample in their entirety and this negates the great advantage of the leaf/stem labelling technique to not require the complete sampling of belowground plant tissues.

For grain legume crops which, as in the case for Brazilian soybean, are highly dependent on BNF for N supply, the large contribution of nodules to belowground biomass N cannot be ignored. In the other studies of rhizodeposited N from soybean nodules were not sampled and there was no information available on the ^{15}N enrichment of nodulated roots and roots without nodules (Rochester et al. 1998; Laberge et al. 2009; Zang et al. 2018). The separation of nodules from roots achieved in our experiments was facilitated by the sandy texture of the soil may well have been impossible in these other studies.

More than any other grain legume, field pea (*Pisum sativum* or *P. avense*) has been the most studied with respect to rhizodeposition of N in both in pot experiments (Jensen 1996b; Sawatsky and Soper 1991; Mayer et al. 2003; Arcand et al. 2013; Hupe et al. 2016; 2018) and in the field (Wichern et al. 2007a; 2007b; Mahieu et al. 2007; Hupe et al. 2019). Most studies mentioned nodulation and/or the contribution from BNF, but no study reported quantitative information on DM, N or ^{15}N enrichment of samples of nodules or nodulated roots compared to roots where nodules had been removed. While it was apparent, and sometimes confirmed, that the dependency of the plants on BNF was high, it would appear

that the failure to quantify the impact of the presence of nodules would lead to large errors the estimates of rhizodeposited N.

Conclusions

1. Within 48 to 72 h after leaf labelling with either ^{15}N-enriched urea or glutamine there was a considerable deposition of tracer N into the soil, which was greater than the tracer present in the roots + nodules. We conclude from the rapid transfer that this N was in a soluble form and the exudation/leakage was an artefact that caused considerable overestimation of rhizodeposited N. The subsequent much slower deposition we conclude was probably derived from root exudates, lysates, sloughed off cells and senescent roots and nodules. Discounting this initial leakage of highly enriched N, on average the estimates of %NdfRh decreased by between 51 and 66%.
2. The mass balance technique relies on supposition that the proportion of all tracer N (in the plant and rhizodeposits) found in the soil is equal to the proportion of all plant N deposited in the soil. Results of several studies, including ours, show that the ^{15}N enrichment of shoot tissue of leaf- or stem-labelled plants is higher than that of root tissue. This leads to an underestimation of rhizodeposited N. The Janzen and Bruinsma (1989) technique relies on the supposition that that all rhizodeposited N is derived from roots and that the roots are uniformly labelled with ^{15}N with time and space. Our results show that after an initial period of less than seven days, root ^{15}N enrichment only changes slowly with time (plant ontogeny) although in this study spatial variation of this parameter was not studied.
3. A large proportion of belowground plant N of soybean was in the nodules which had a lower ^{15}N enrichment than any of the root cohorts. On average for all three experiments the estimates of %NdfRh discounting the initial tracer leakage increased from 7.2% for the estimate based on the ^{15}N enrichment of clean roots to 10.1% for that based on the nodulated roots to 23.4% when based on ^{15}N enrichment of nodules. Difficulties in recovering a representative sample with the "correct" proportion of root and nodule tissue can be a major source of error in the quantification of rhizodeposited N.

Acknowledgements The authors thank Alderi F. da Silva, Aurelio de S. Chagas, Cláudio P. Ferreira, Enivaldo Maia, Ernani C. de Meirelles and Roberto C. da S. Ramos for help in setting up and tending to the plants and to Dr Renato M. da Rocha for meticulous work on the isotope-ratio mass spectrometers. The authors KECA, CV, RCS and WMS gratefully acknowledge postgraduate fellowships from the Ministry of Education (CAPES) and SU, BJRA and RMB fellowships from the National Research Council (CNPq) and grants (Cientista de Nosso Estado) from the Rio State Research Foundation (FAPERJ). The work was funded by CNPq (Grant No. Processo No. 305047/2016-7), FAPERJ (Grant No. Processo No. E-26/203.031/2017) and Embrapa.

Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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