

RESEARCH ARTICLE

# An isotopic proxy for nitrogen redistribution from *Alnus acuminata* to wheat intercrop

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## Summary

Direct belowground nitrogen (N) transfer has often been reported where plants with contrasting nutrients acquisition strategies (N<sub>2</sub>-fixing and non-fixing) co-occur, and there is still a gap in the knowledge of the extent of this transfer in the top soil under the field conditions. However, assessment under field conditions is challenging. We hypothesized a practical application of the analysis of natural abundance of δ<sup>15</sup>N supplemented with an isotopic mixing model 'IsoSource' to understand the relative direct contribution of N<sub>2</sub>-fixing *Alnus acuminata* to wheat intercrop (non-fixing) N isotopic signatures. A field experiment was conducted in an andic soil of high lands in northern Rwanda to quantitatively determine the proportional contribution of nitrogen by *Alnus acuminata* to wheat vegetative tissue isotope signatures at different distances from the trees (1 m, 3 m, 5 m, and 7 m). The study involved the measurements and analyses of natural abundance of stable isotopes δ<sup>15</sup>N and isotopic mixing modeling by IsoSource. Leaf samples were collected from twigs of 10 years old *Alnus acuminata* grown on the terrace-risers, along with soil samples (0–20 cm) and wheat flag leaf samples across terrace at 1 m, 3 m, 5 m, and 7 m from trees for isotopic measurement. The chlorophyll content index of wheat flag leaf at the four points across terrace was estimated by means of SPAD meter 502. The δ<sup>15</sup>N proportional contribution by *Alnus acuminata* to wheat was obtained through IsoSource analysis. We noted a significant ( $p < 0.01$ ) gradient in depletion of wheat δ<sup>15</sup>N signatures moving further away from the tree line of *Alnus acuminata*. The wheat at 1 m from the trees exhibited the δ<sup>15</sup>N values closer to that of the tree, while at 7 m, the crop δ<sup>15</sup>N signature was significantly different from that of the tree. An isotopic mixing model 'IsoSource' indicated that the tree N may have provided  $33.6 \pm 4.3$  % of the wheat intercrop N at 1 m distance from the trees. Therefore, this study shows that the understanding of field-based crop N and nutrient transfer in agroforestry may be enhanced by analysis of the physiological basis of stable isotopes signatures.

**Keywords:** Intercropping; Isotopic proxy; N transfer; IsoSource; Wheat; *Alnus acuminata*; Rwanda

## Introduction

The practice of agroforestry on agricultural land has been the subject of considerable research (Akinnifesi *et al.*, 2010; Schroth, 1998; Luedeling *et al.*, 2016; Dupraz *et al.*, 2019; Sida *et al.*, 2020; Cardinael *et al.*, 2019b; Clivot *et al.*, 2020). Trees modify soil properties through numerous processes (Rhoades, 1997; Isaac and Borden, 2020; van Noordwijk *et al.*, 2019). Tree and crop root systems play a crucial role in the belowground interactions (Bayala and Prieto, 2020; Bardgett *et al.*, 2014). Biological nitrogen fixation by tree, crop, or both components of agroforestry systems has received a lot of attention in the tropics (Nair *et al.*, 1999). There is a sense that resource use is increased under agroforestry systems (Vandermeer *et al.*, 1998); for example, belowground transfer of nitrogen among plants has been reported, from a N<sub>2</sub>-fixing source plant to a non-fixing plant sink (Haystead *et al.*, 1988; Arnebrant *et al.*, 1993; He *et al.*, 2009). The nitrogen transfer

from  $N_2$ -fixing trees can be a major source for the associated crops in a low-inputs farming system. According to Lal (2004), the N is the most important nutrient limiting crop production in the tropical small-scale farming; therefore integrating the  $N_2$ -fixing trees into farmland may improve the N supply of the cropping system.

Plants are known to exude nitrogenous compounds from living roots (Jalonen *et al.*, 2009). These compounds can be absorbed as such by adjacent plants without prior mineralization by soil microorganisms (Jones *et al.*, 2004). Several mechanisms involving N transfer from  $N_2$  fixation by plants have been reported by different authors, via: roots grafts or root-to-root contacts (Caldwell and Richards, 1986); roots exudations (Teste *et al.*, 2014); and mycorrhizal networks (He *et al.*, 2003; Barea *et al.*, 2005; Battie-Laclau *et al.*, 2020). But it is not clear how important it is quantitatively. It is also argued that after root exudation, two basic mechanisms may be transporting ions and simple amino acids to neighboring plants; the first is mass flow that moves ions along with the flow of water and the second is the diffusion of ions along concentration gradient without the flow of water (Teste *et al.*, 2014).

According to Evans (2001), whole plant and leaf N isotope composition is determined by the isotope ratio of the external N sources and physiological mechanisms within the plant. The physiological mechanisms that influence plant N isotopic signature have been reviewed by Evans (2001), and earlier by Högberg (1997) and Handley and Raven (1992).

The study of N transfer among plants could have potential in agro-farming under low external inputs (Jensen, 2005; Wichern *et al.*, 2008). Scientists initially hoped that quantifying  $\delta^{15}N$  could be used to trace the relative contribution of  $N_2$  fixation to plants and soils (Michener and Lajtha, 2007). Many authors have used  $\delta^{15}N$  data to draw inferences regarding N sources (Garten *et al.*, 2007; Evans *et al.*, 2007; Phillips and Greggs, 2003). For example, Schulze *et al.* (1991) employed the natural abundance of  $\delta^{15}N$  values in Acacia savannas to estimate the nitrogen fixation by the *Acacia melifera* trees on aridity gradient in Namibia, and found that about 71 % of nitrogen was fixed. Similarly, Shearer *et al.* (1983) used the natural abundance of  $\delta^{15}N$  in tissues of *Prosopis grandulosa* to estimate the  $N_2$ -fixation by these trees, and concluded it is feasible to use variation in natural abundance of  $\delta^{15}N$  as an index of  $N_2$ -fixation. Additionally, studies in the California Sonoran desert indicated *Prosopis* woodland fixed a significant amount of  $N_2$  based on soil N accumulation beneath (Virginia and Jarrell, 1983); since desert soil is often deficient in nitrogen, they argued that  $N_2$ -fixing by *Prosopis* tree might be the basis of that soil N accumulation. However, the novel in this research is coupling the isotopic mixing modeling “IsoSource” to the plant natural abundance of  $\delta^{15}N$  to determine the proportional contribution of N by  $N_2$  fixing plant to a non-fixing intercrop.

There would be a great deal to be learned from  $\delta^{15}N$  of plant tissues and their sources of N. The aim of this research was to experiment if the natural abundance of  $^{15}N$  coupled to isotopic mixing modeling can be applied to determine the relative transfer of N from a  $N_2$  fixing tree to a non-fixing intercrop in the field.

## Material and methods

### Description of research area

The research described here was conducted at three sites located in Northern Rwanda (Supplementary Material Fig. S1). The site at Rurembo was located at 01° 53' S; 29° 57' E and elevation 2245 m. The second site at Kirezi was at an elevation of 2269 m and at 01° 54' S; 29° 57' E. The third site at Cyansure was located at 01° 55' S; 29° 57' E, and elevation of 2248 m.

The rain distribution across the whole region is bimodal, characterized by long and short rain seasons that allow two cropping seasons a year. Based on climatic data of the local weather station in the study area, the average annual rainfall during the experiment was 1640 mm, with an average annual temperature of 15 °C. The soils of the area are Alfisols (*Ultic Tropudalf*), Inceptisols (*Andic*

*Eutropept*; *Typic Dystrandept*; *Entic Eutrandept*), and Mollisols (*Cumulic Hapludoll*) (Supplementary Material Fig. S2). The pH (in water) of the soil was 6.7, and the dominant crops are wheat, potatoes, maize, and peas. Potatoes and wheat are the main cash crops in the area.

### Experimental setting and data collection

The experiment was conducted on the farmers' fields' terraces that were established in 2010 with transplants of *Alnus acuminata* trees on the terrace risers (Supplementary Material Fig. S3). The spacing between trees was 5 m, and the terrace width was 8 m. The trees on the terrace risers were pruned three times a year. Wheat (variety Bisagi) was grown as a test crop on terrace's bench in all fields of the study. The planting density for wheat was 150 plants m<sup>-2</sup>.

### Sampling and measurement

Thirty leaves per field (90 for the whole experiment) were collected on the twigs of *Alnus acuminata* trees for δ<sup>15</sup>N measurement. Soil was sampled using a soil auger at the depth of 0–20 cm in each plot at four points across the terrace's bench at 1 m, 3 m, 5 m, and 7 m from the trees, and three replicates. A total number of 40 composite soil samples were taken, and air-dried and sieved to less than 2 mm for δ<sup>15</sup>N determination. At anthesis (GS65), the flag leaf wheat sample (144 in total) was collected at four points further away from the trees (1 m, 3 m, 5 m, and 7 m) in the bench along the slope and three replicates for measurements of specific leaf area (SLA), and Carbon discrimination (Δ<sup>13</sup>C) and δ<sup>15</sup>N. At each of the four points, the chlorophyll content index (CCI) of the flag leaf was measured using the chlorophyll meter SPAD-502 (Konika Minolta Sensing Inc., Osaka, Japan). SLA (cm<sup>2</sup> g<sup>-1</sup>) was obtained by measuring the leaf area (LA), one side of the leaf, with *ImageJ* (version 1.42q, National Institute of Health, USA). Then, the sample was oven-dried in a paper envelope at 75 °C for 24 h. The leaf dry weight (DW) was obtained by reweighing the sample on micro-balance after oven drying.

The SLA was calculated as the ratio of LA to DW:

$$\text{SLA}(\text{cmg}^{-1}) = \frac{\text{LA}(\text{cm}^2)}{\text{Dw}(\text{g})} \quad (1)$$

For the isotopic measurement and analysis, the dry leaf and soil samples were ground separately using ball mill (MM200 Mixer Mill, Glen Creston Ltd, UK). For each sample, a subsample of 1 mg for leaf and 10 mg for soil was weighed into a tin capsule and analyzed for δ<sup>15</sup>N at the Godwin Laboratory (University of Cambridge) using a Costech elemental analyzer attached to a Thermo Delta V mass spectrometer in continuous flow mode. The δ<sup>15</sup>N data were analyzed for the proportional contribution sources to the crop <sup>15</sup>N signatures, using an isotopic mixing model 'IsoSource' (Phillips and Gregg, 2003).

The Carbon discrimination (Δ<sup>13</sup>C) was obtained through the measurement of the value of δ<sup>13</sup>C in the dried ground leaf samples weighed (1 mg) into a tin capsule, using Costech elemental analyzer attached to a Thermo Delta V mass spectrometer in continuous flow mode. The δ<sup>13</sup>C value was used to compute the Δ<sup>13</sup>C following Farquhar *et al.* (1982):

$$\Delta^{13}\text{C} = \left( \frac{\delta^{13}\text{C}_a - \delta^{13}\text{C}_p}{1 + \delta^{13}\text{C}_p} \right) / 1000 \quad (2)$$

where the δ<sup>13</sup>C<sub>a</sub> is the delta value of C in the air and the δ<sup>13</sup>C<sub>p</sub> is the delta value of C in the sample.

### Statistical analysis

The statistical analysis of the data was performed using SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA). Firstly, the data were explored for parametric assumptions of normal distribution and homogeneity of variance using Kolmogorov–Smirnov (*K–S*) and Levene's tests, respectively.

The graphing of means was performed using bar charts, and the data were subjected to one-way independent ANOVA at  $p < .01$ , followed by Bonferroni test at significance level  $p < .01$ .

## Results

The  $\delta^{15}\text{N}$  of the wheat intercrop indicated consistent gradient declining with the distance from the  $\text{N}_2$  fixing *Alnus acuminata* trees (Table 1). The wheat nearest to trees of *Alnus acuminata* showed the  $\delta^{15}\text{N}$  signature values closer to that of the tree; at 1 m from the trees, the wheat signature was  $7.23 \pm 0.52$  ‰ relative to the tree ( $7.50 \pm 0.13$  ‰), while further at 7 m, the crop  $^{15}\text{N}$  signature was  $3.22 \pm 0.83$  ‰. The isotopic mixing model indicated that tree N could theoretically have provided  $33.6 \pm 4.3$  % of the crop N at 1 m (Figure 1a). Additionally, the CCI of wheat leaf was significantly higher at 1 m, 3 m, and 5 m from the trees, which declined moving further out into the bench (Figure 1b). The results also revealed a gradient decline both in the value of SLA and  $\Delta^{13}\text{C}$  of wheat leaf intercrop toward the tree lines of *Alnus acuminata* (Figure 1c, d).

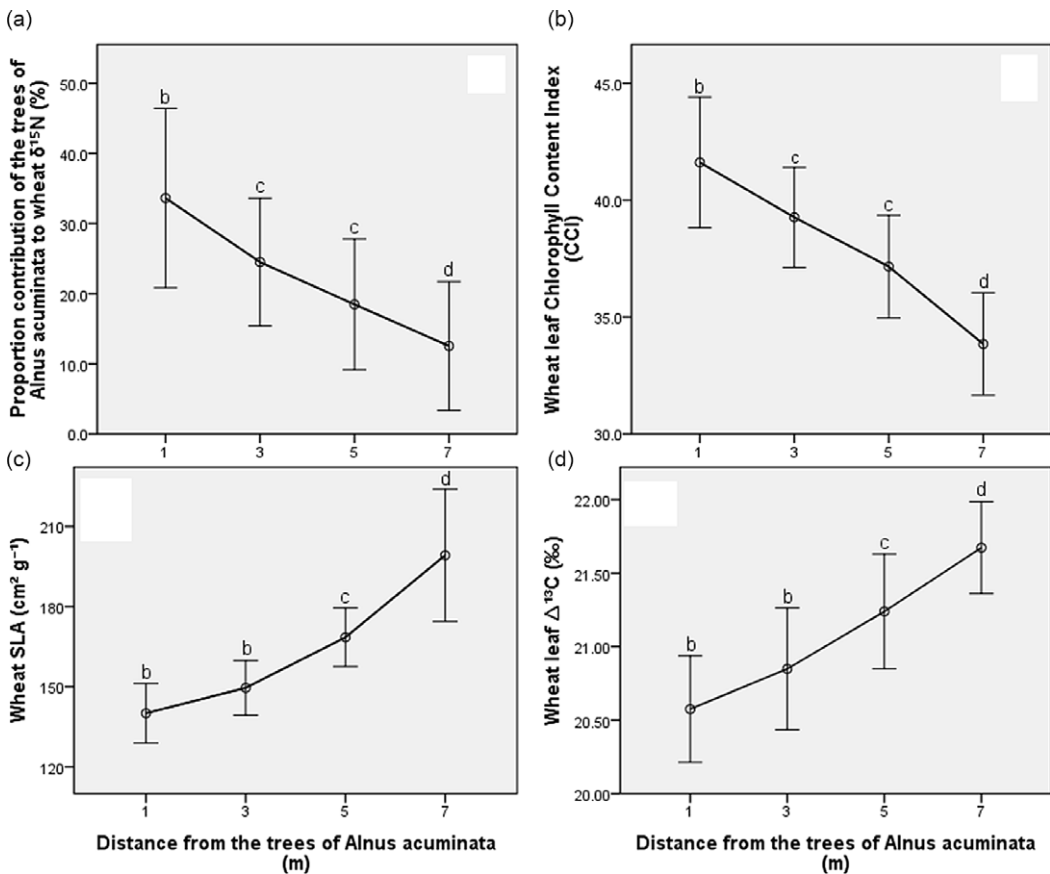


Fig. 1. The proportional contribution of *Alnus acuminata* to wheat intercrop N (a), Chlorophyll content of wheat leaf (b), Specific leaf area of wheat leaf (SLA, in c) and  $\Delta^{13}\text{C}$  of wheat leaf (d) at different distances from the *A. acuminata* trees across the bench terrace. Values are means  $\pm$  SE ( $N$  144). The bars showing the same letters indicate that their mean values do not statistically differ significantly ( $p < 0.01$ ).

**Table 1.** The mean isotopic values of wheat leaf at different distances from the *Alnus acuminata* trees across the bench terrace and of the sources (*Alnus acuminata* tree; soil) of N

Isotope	Distance from tree (m)	$\delta$ value of crop (mean $\pm$ se)	
		N = 144	
		$\delta$ Source (mean $\pm$ se)	
$\delta^{15}\text{N}$	1	Tree: 7.50‰ $\pm$ 0.13 (N = 90)	
	3	Soil (0–20 cm): 1.38 ‰ $\pm$ 0.02 (N = 40)	
	5	7.23‰ $\pm$ 0.52	
	7	6.62‰ $\pm$ 0.77	
		5.98‰ $\pm$ 0.62	
		3.22‰ $\pm$ 0.83	

## Discussion

The understanding of the physiological basis of isotope signatures of the plant nitrogen may be an approach to encapsulate the plant interaction and resources acquisition in agroforestry systems. The key to this research was that the distinct isotopic signatures of various sources of plant nitrogen can be identified, and their relative contribution to plant N could be determined by an isotopic mixing model ‘IsoSource’. The results indicated that *Alnus acuminata*, a  $\text{N}_2$ -fixing tree, exhibits nitrogen transfer.

The transfer of nitrogen has been reported to exist where plants with contrasting nutrients acquisition strategies ( $\text{N}_2$ -fixing and non-fixing) co-occur (Dawson *et al.*, 2002; He *et al.*, 2009). However, there remains controversy about whether belowground N transfer occurs (Ikram *et al.*, 1994; Johansen and Jensen, 1996); and often research on N transfer in agroforestry has conventionally assumed that N transfer occurs via the decomposition of legume litter and pruning residues in soil (Jalonen *et al.*, 2009).

We addressed these questions with the assessment of natural abundance of  $\delta^{15}\text{N}$  and an isotopic mixing model to determine the proportional contribution of N by *Alnus acuminata* to the wheat intercrop N. The literature has been highlighting the need to study N transfer among plants toward agro-farming under low external inputs (Hauggaard and Jensen, 2005; Wichern *et al.*, 2008); therefore we aimed to inform the design of intercropping of  $\text{N}_2$  fixing trees with wheat as an intercrop for enhanced transfer and distribution of N to the non-fixing  $\text{N}_2$  intercrop.

The data from this study provided an indication that N transfer from  $\text{N}_2$ -fixing trees can be a considerable N source for the associated intercrop in agroforestry farming (Figure 1a and Table 1). *Alnus* have been reported to have the potential to provide N-fixation benefits in temperate agroforestry systems (Sharifi *et al.*, 1982; Seiter *et al.*, 1995). Seiter *et al.* (1995) demonstrated this potential in a red alder (*Alnus rubra* Bong.) – maize alley cropping system in Oregon.

Similarly, in this study, a comparison of the  $\delta^{15}\text{N}$  signatures of the tree and the wheat revealed that the crops in the proximity of trees exhibited value closer to the tree  $\delta^{15}\text{N}$  and declined as moving further in the terrace (Table 1), and these are supported with the value of both SLA and  $\Delta^{13}\text{C}$  wheat intercrop. In agreement with our data, Seiter *et al.* (1995) observed, using an  $^{15}\text{N}$  injection technique, that 32–58% of the total N in maize was obtained from  $\text{N}_2$  fixed by red alder and that nitrogen transfer increased by shortening the distance between the trees and crops. Our results are also consistent with the works of many authors (Handley and Scrimgeour, 1997; Robinson, 2001; Evans, 2001; Stewart, 2001) who suggested that the  $\delta^{15}\text{N}$  of leaf tissues reflect the net effect of  $\delta^{15}\text{N}$  of the sources used by that plant. Our findings are in accordance with the works of both Moyer *et al.* (2006) and Lu *et al.* (2013) who showed that N transfer among plants can occur through the release of N compounds from the  $\text{N}_2$ -fixing plant leading to uptake by a non  $\text{N}_2$ -fixing plant. The relative N transfer of  $33.6 \pm 4.3\%$  at 1 m from the trees observed in this study agrees with Sierra and Daudin (2010) who assessed in situ the  $^{15}\text{N}$  transfer from stem-labeled trees to associated grass and found that the transfer of the added  $^{15}\text{N}$  was limited in space (up to 1 m from trees) and was on average 33%. Similarly, Snoeck *et al.* (2000) noted 13–42% of  $^{15}\text{N}$  transfer from the legume trees to coffee. These findings are also in agreement with Handley and Raven (1992) who suggested that there is no evidence of

fractionation either of  $\delta^{14}\text{N}$  or  $\delta^{15}\text{N}$  during its physical movement (passive and active uptake) across living membranes of plants.

Nevertheless, such data should be interpreted with caution; for example, Daudin and Sierra (2010) observed that grass presented a preferential uptake of N released by the tree; if that is the case, then this preferential N uptake may cause discrepancy in isotopic mixing model results. Similarly, Sierra *et al.* (2007) argued that N transfer from  $\text{N}_2$  fixing trees may involve direct and indirect pathways; i.e. N transfer could be indirect if N exudates from the roots of tree were taken by soil microorganisms and passed through microbial turnover (Høgh-Jensen, 2006); in that case, the isotopic mixing model could not resolve such system because it takes into account only N sources. The pruning regime (frequency and intensity) was also argued to be another factor that may affect N transfer by limiting the rate of  $\text{N}_2$  fixation (Nygren *et al.*, 2000).

Moreover, Sanchez *et al.* (1997) argued that the roots of trees are often able to capture nutrients at the depths beyond the reach of most crop and redistribute them into topsoil, and this can be an additional nutrients input in an agroforestry system.

Finally, the question of which mechanisms (and their importance) that drive the N transfer between plants remains unsolved up to date; several mechanisms have been reported; release of N in exudates (Høgh-Jensen, 2006), roots-grafts (Caldwell and Richards, 1986), and mycorrhizal networks (He *et al.*, 2003), but the extent of their contribution to N transfer remains unsolved. We, therefore, recommend further research into the molecular mechanisms by which plants transfer N to their neighbors.

**Supplementary material.** To view supplementary material for this article, please visit <https://doi.org/10.1017/S0014479721000284>

**Conflict of interest.** None.

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