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RESEARCH ARTICLE

Nitrogen fixation of lablab and finger millet in South-India

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Abstract

Background: In a long-term rotation experiment (2016–2022) with different nitrogen (N) fertilizer levels in subtropical South-India, crop yields of low N plots were unexpectedly high. We therefore hypothesized that in the absence of mineral N application, these yields are largely due to N inputs by N_2 fixation in the component crops. To assess the diazotrophic N_2-fixation of lablab (*Lablab purpureus* L. Sweet) and possible associative N_2-fixation of finger millet (*Eleusine coracana* L. Gaertn), a controlled experiment was conducted during the 2021 monsoon season within the above-mentioned long-term field study. Two approaches were used to estimate the quantity of N derived from the atmosphere (Ndfa): the dilution method using a ^{15}N-labeled fertilizer and the natural abundance method.

Method: For the ^{15}N dilution method irrigated maize (*Zea maize* L.), finger millet and lablab were labeled with two split applications of 10% ^{15}N fertilizer (50:50 ^{15}N-urea and ^{15}N-ammonium sulfate) amounting to a total of 15 kg N ha^{-1}. Maize was selected as the non-fixing reference plant to estimate diazotrophic N_2-fixation. The entire aboveground biomass of the labeled plants was harvested at maturity and analyzed for total DM, N concentration, and the ^{15}N isotope ratio.

Results: N_2 fixation efficiency for lablab was 52%–69% depending on the calculation method, corresponding to 40–53 kg N ha^{-1}. For finger millet, the natural abundance method resulted in an estimated N_2-fixation of 5 kg N ha^{-1}, which was suggested by the results of the dilution method whereby the reference plant maize was only poorly labeled.

Conclusion: Labeling of maize might have been diluted due to unexpected associative N_2-fixation or N-uptake from unlabeled deep soil N pools. The data underline the importance of symbiotic N_2-fixation in crop rotation systems of South-India.

KEYWORDS
^{15}N, dystric nitisol, *Eleusine indica*, isotopic labeling, *Lablab purpureus*, symbiotic and associative N_2-fixation

1 | INTRODUCTION

Over the last two decades, in Bengaluru, South-India, one of India's megacities with a population exceeding 13 million inhabitants, pressure on land use for living space, local recreation, infrastructure, and, above all, food production grew rapidly. To investigate how the intensification of agricultural land use as a consequence of rural–urban transformation affects crop yields, matter balances, and soil quality, a factorial crop rotation experiment was established in 2016 at the Gandhi Krishi Vigyana Kendra (GKVK) campus of the University of Agricultural Sciences Bangalore (UASB).

After 7 years (2016–2022), total dry matter (TDM) data of the locally typical experimental crops maize (*Zea maize* L.), finger millet (*Eleusine coracana* Gaertn.), and lablab (*Lablab purpureus* L. Sweet) grown in the rainy season from July to December (*Kharif*) and tomato/chilli (*Solanum lycopernicum* L./*Capsicum* L.), eggplant (*Solanum melongena* L.), and cabbage (*Brassica oleracea* L.) in the dry season from February to June (*Rabi)* (Hoffmann et al., 2021) showed a remarkable pattern. Although even at high levels of nitrogen (N) and other mineral fertilizers, overall TDM yields declined over time, crops in zero N plots continued to achieve notable yields (Buerkert et al., 2023). This study was therefore conducted to determine to what degree symbiotic N_2-fixation by lablab in a crop rotation and associative N_2-fixation by finger millet have contributed to N-nutrition of the crops grown.

There are two different methods to quantify N_2-fixation, which have their advantages and disadvantages. The isotope dilution method is known to yield reliable but costly results that allow to quantify the amount of N_2-fixation by pulse labeling soil N pools with a high ^{15}N dose (Fenilli et al., 2007; He et al., 2009; Sarr et al., 2016). Effective use of this approach requires a constant and homogeneous ^{15}N-labeling in the rooting zone across the entire cropping season (Khan et al., 2003). Crops grown on the labeled soil quickly take up N as ammonium released from labeled ammonium sulfate (Braun et al., 2018) and nitrate released from urea after microbial turnover. In plants without N uptake from associative or symbiotic N_2-fixation, the increased $^{15}N/^{14}N$ ratio in plant biomass N resulting from soil N uptake is diluted by natural abundance N fixed from air N_2. The $^{15}N/^{14}N$ ratio in atmospheric N_2 is lower than in labeled soil mineral N pools; hence, the more N plants take from the atmosphere, the lower their $^{15}N/^{14}N$ ratio. For the quantification of N_2 fixation a non-fixing reference crop is needed, which obtains its N only from the soil N pools. Ideally, this reference crop has a similar rooting zone and N uptake pattern as the investigated N-fixing crop (Bremer et al., 1993). Maize is a widely used reference plant and usually does not contribute to associative N_2-fixation, although a few traditional varieties were reported to display some associative N_2-fixation (Montañez et al., 2009; Palus et al., 1996). The natural abundance method instead does not require artificial labeling of the soil N pool but uses the difference of the natural abundance of ^{15}N and ^{14}N in mineral soil N and atmospheric N_2. The latter has a lower $^{15}N/^{14}N$ ratio than plant available soil N pools. This difference can be used to calculate the proportion of N uptake by a crop with diazotrophic N_2-fixation.

2 | MATERIALS AND METHODS

2.1 | Experimental site

A crop rotation experiment at the GKVK campus of UASB with a randomized factorial design established in 2016 comprised 12 main experimental plots (12 × 18 m^2) whereby each plot was divided into three subplots (12 × 6 m^2) for three N-fertilizer levels (low, medium, and high). Within these 36 plots crops grown typically by regional farmers, maize , finger millet , and lablab were grown annually in the rainy season from July to December in a 3-year crop rotation. In successive seasons, lablab, maize, and finger millet were rotated, whereas the N-fertilizer status in the subplots remained fixed for the entire duration of the experiment (Buerkert et al., 2023). The ^{15}N-tracer experiment was performed in 2021 within this crop rotation experiment in the plots with zero N fertilizer (termed "Low N" plot) amendments of the irrigated factorial crop rotation experiment.

The low fertilizer plots did not receive any fertilizer inputs after January 2018. The local soil is classified as a typical, weathered Kandic Paleustalf/dystric Nitisol according to the USDA/FAO classification, with a medium pH of 6.91 at 0–10 cm depth and 6.55 at 10–30 cm, containing 56% sand, 9% silt, and 33% clay. The average total N concentration in the upper layer was 0.74 and 0.83 mg g^{-1}, and 0.80 and 0.71 mg g^{-1} in the lower layer before and after the experiment, respectively. The addition of 15 kg N ha^{-1} with 10% ^{15}N resulted in an estimated labeling of the soil N pool of 0.442 at% ^{15}N. Climate data at the experimental site were recorded by a Hobo weather station (H21-002; Onset Inc., Bourne, MA, USA) at 1.5 m above the field with sensors for air and soil temperature, soil moisture, rainfall, and relative humidity recording at 10 min intervals. The mean maximum temperature of the season was 27.9°C at noon and the mean minimum temperature 19.5°C during early morning hours. Rainfall was typical for the *Kharif* season in Bangalore with a cumulative value of 646 mm during the duration of the experiment (Figure 1). Precipitation events after the first and second fertilization dates were moderate and thus unlikely to have caused significant leaching of the surface-applied ^{15}N marker beyond the rooting depth or outside of the wooden delineation frames established around the micro-plots.

2.2 | Micro-plots and ^{15}N-tracer application

To quantify biological N_2-fixation by finger millet and lablab, a ^{15}N-tracer experiment was performed in micro-plots of 2.2 × 1.3 m^2 in each of the four replications of the experiment. The micro-plots were set up using wooden frames of 20 cm height, which were embedded 5 cm deep into the soil near the center of the experimental macro-plots of 6 × 12 m^2 after seedbed preparation (Figure 2). This facilitated homogenous stand growth with minimal border effects.

On 25.8.2021, maize and lablab were planted at 20 cm distance in rows 60 cm apart, whereas finger millet was planted at 9 cm in rows

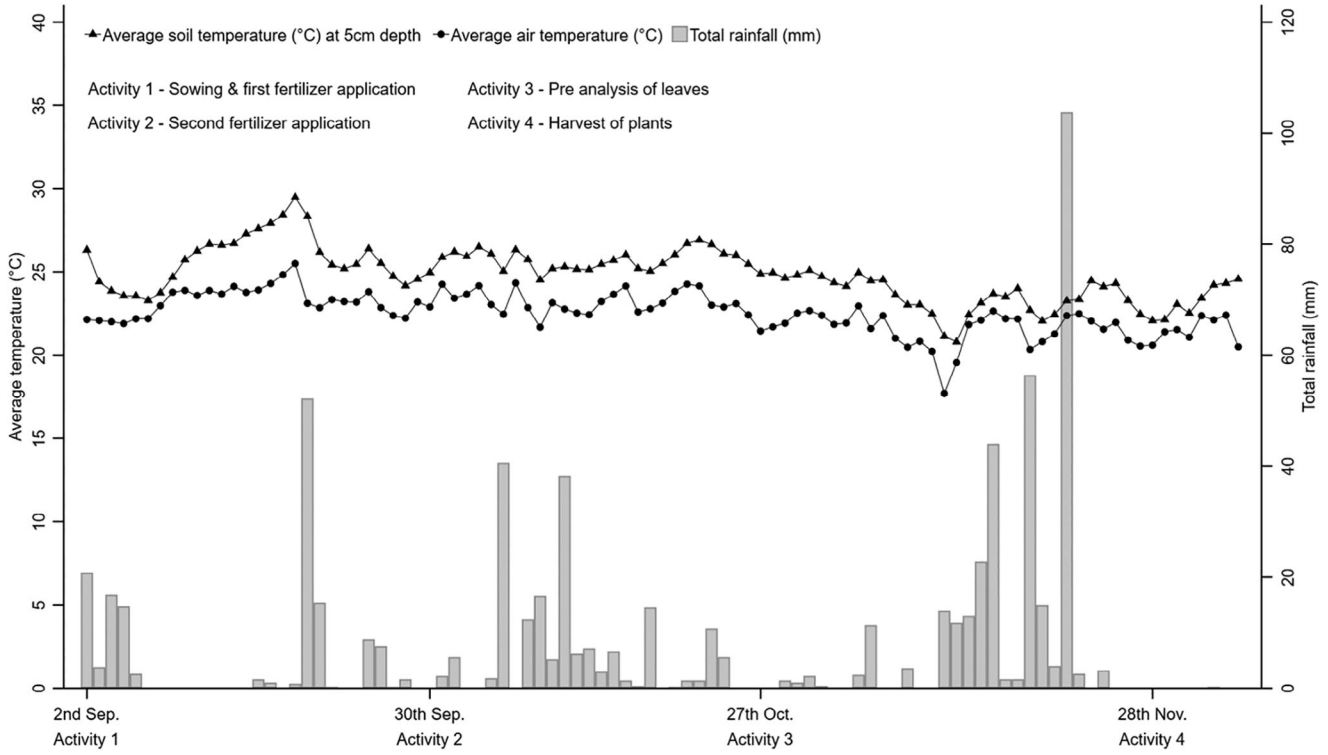

FIGURE 1 Average daily soil temperature at 5 cm depth in °C, average daily air temperature in °C, total rainfall per day in mm and field activities of sowing (A1), split fertilizer applications (A1 + A2), first sampling for pre-analysis (A3), and final harvest of labeled plants (A4) of a ^{15}N tracing experiment in an irrigated field experiment at the GKVK Campus of the University of Agricultural Sciences Bangalore (UASB), Bengaluru, South India.

distanced 30 cm. The micro-plots thus covered 4 rows with 22–33 plants each for maize and lablab, and 7 rows with 93 plants for finger millet. On 2.9.2021 and 30.9.2021, ^{15}N was applied as a mixture of urea (CH_4N_2O; Art.No. CS01-185_304; Campro Scientific GmbH, Berlin, Germany) and ammonium sulfate $(NH_4)_2SO_4$; Art.No. NB-6019, Chemotrade Chemiehandelsgesellschaft mbH & Co. KG, Duesseldorf, Germany) in two split applications at a rate of 7.5 kg ^{15}N ha^{-1} per application. During each application, 2.3 g ^{15}N-urea (10 at% ^{15}N) and 3.9 g ^{15}N-ammonium sulfate (10 at% ^{15}N) were dissolved in 2 L of deionized water and sprinkled onto the soil surface of the micro-plots using a calibrated watering can. Except for the application of ^{15}N tracer and crop-specific amounts of phosphate and potassium, micro-plots did not receive any fertilization and were treated as the macro-plots.

2.3 | Harvesting and analysis

On 27.10.2021, 63 days after sowing (DAS), 2–3 leaves were taken from border plants of each micro-plot to allow the verification of successful plant labeling. On 28.11.2021, near maturity, the aboveground biomass of all crops was harvested. In another effort to minimize border effects on ^{15}N-signature dilution from surrounding soil, for lablab and maize, only the inner 8–12 plants and for finger millet the inner 41–73 plants of the micro-plots were harvested separately. The harvested plants were carefully cleaned from adhering soil contamination, separated in generative (cob and corn) and vegetative parts (shoot and leaves) weighed, and chopped in smaller pieces for drying at 60°C to constant weight. Utmost care was taken to avoid cross-contamination of the samples. The completely dried samples were pooled plot-wise and transported to Germany for further processing and isotope analysis. After pre-cutting with scissors and crushing with a Retsch mill (SM1, Retsch GmbH, Haan, Germany), used exclusively for maize and finger millet, the samples were re-dried at 60°C and then ground to 0–2 mm with a laboratory mill (CT 193, Foss Analytical A/S, Hillerod, Denmark). All processes were carried out with as little losses as possible and the use of a standardized sample divider ensured homogeneous distribution of individual plant constituents in subsamples of vegetative and generative plant materials. To avoid cross-contamination between samples during milling, all equipment were cleaned with 70% ethanol between samples, and plant materials with lowest estimated labeling were processed first. After another re-drying at 60°C, biomass samples of labeled and control plants containing 1.5 mg of lablab, 2.0 mg of maize, and 3.0 mg of finger millet were weighed with an ultra-precision balance (Sartorius AG, Göttingen, Germany) into tin capsules and sent to the laboratory of the Competence Center Stable Isotopes (Büsgen Institute, Georg-August-Universität Göttingen) for analysis by stable isotope mass spectrometry (Conflo III, Delta C, Thermo Fisher Scientific GmbH, Dreieich, Germany) and elemental analyzer (Euro EA 3000, EuroVector S.p.A, Milan, Italy). Using a similar proce-

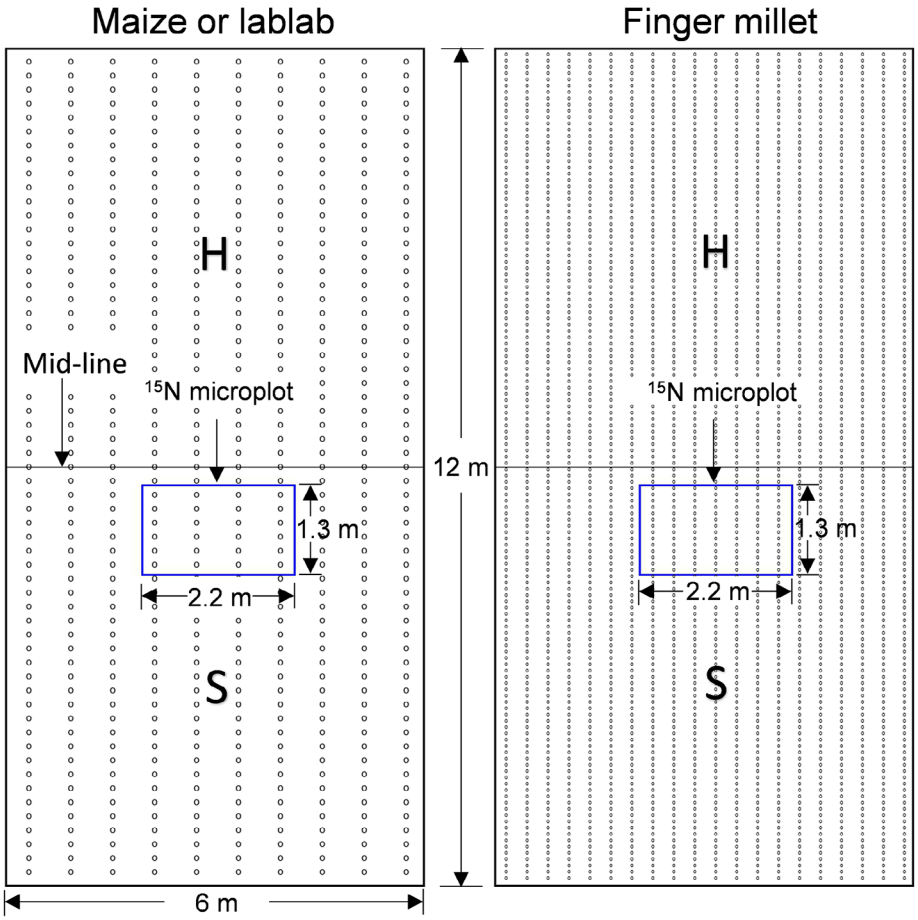

FIGURE 2 Position of the ^{15}N-micro-plots in a crop rotation experiment with maize, finger millet, and lablab at the GKVK Campus of the University of Agricultural Sciences Bangalore (UASB), Bengaluru, South-India. H and S subplots indicate two identical plot parts with different physiological measurements during the cropping season.

dure, labeled and unlabeled soils were analyzed using 9.0 mg per tin capsule.

The pH (1:2.5 m:vol in 0.01 M $CaCl_2$) of the post-experimental soil was determined in five pooled samples from 0–10 cm to 10–30 cm depth sieved to 2 mm (pH3110, WTW, Xylem Analytics Germany Sales GmbH & Co. KG, Weilheim, Germany).

2.4 | Calculations and statistical analysis

For the dilution method (*dil*), the N derived from ^{15}N fertilizer (Ndff), N derived from the atmosphere (Ndfa), and the N derived from the soil (Ndfs) were estimated using the equation of the International Atomic Energy Agency (Sarr et al., 2016):

$$\%\text{Ndff} = \frac{^{15}\text{N atom\% excess labeled plant}}{^{15}\text{N atom excess fertilizer}} \times 100, \quad (1)$$

whereby *^{15}N atom% excess* is the difference of the ^{15}N atom% of the labeled sample or fertilizer and the respective natural ^{15}N abundance. Using the %Ndff of the N_2 fixing crop and the reference crop, %Ndfa was calculated as

$$\%\,\text{Ndfa}_{\text{dil}} = \left(1 - \frac{\%\text{Ndff}_{\text{fix}}}{\%\text{Ndff}_{\text{REF}}}\right) \times 100, \quad (2)$$

whereby *%Ndff$_{fix}$* is the %Ndff of the N_2 fixing plant, and *%Ndff$_{REF}$* is the %Ndff of the reference plant. %Ndfs was calculated as the difference of 100%—%Ndff for the reference plant and 100%—%Ndff—%Ndfa for N_2 fixing plants. Quantities of N derived from the different sources were determined by multiplying %Ndff, %Ndfa, and %Ndfs with the total N uptake of the aboveground dry matter in kg ha^{-1}. N use efficiency (NUE) was calculated by dividing Ndff (kg N ha^{-1}) by the N input from the labeled fertilizer (kg N ha^{-1}).

For the natural abundance method (nat), %Ndfa was calculated as (Unkovich et al., 2008):

$$\%\text{Ndfa}_{\text{nat}} = \left(1 - \frac{\delta^{15}\text{N fixing plant}}{\text{average }\delta^{15}\text{N reference plant}}\right) \times 100, \quad (3)$$

using the δ^{15}N obtained from ^{15}N analysis of aboveground biomass of unlabeled plants and dividing it by the average δ^{15}N of the reference plant maize.

TABLE 1 $\delta^{15}N$ ‰ values of soil and crop biomass at the end of a ^{15}N-tracer experiment at the GKVK Campus of the University of Agricultural Sciences Bangalore, Bengaluru, South-India.

					p-Value		
	Maize	**Finger millet**	**Lablab**	**CV%**	**Crop**	**Depth/part**	***C* × *D/P***
Unlabeled soil	5.7						
Labeled soil 0–10 cm	94.2	128.0	96.3	18.1	0.08*	<**0.001**	0.21
Labeled soil 10–30 cm	20.3	22. 8	25.0	24.6			
Unlabeled crop biomass							
Vegetative	15.6	7.7	5.0	41.4	0.16	0.58	0.59
Generative	10.2	5.6	7.3	46.0			
Labeled crop biomass							
Vegetative	1925.9 **a**	2887.5 **a**	848.0 **b**	14.2	<**0.001**	**0.002**	**0.002**
Generative	1652.4 **a**	2476.4 **a**	959.3 **b**	20.3			

Note: Data show means ($n = 4$) with their mean coefficient of variation (CV%) and *p*-values of a mixed model using plot as subject, nested in depth (for soil) and parts (vegetative and generative for crops) and with crops, depth/parts, and their interaction as fixed factors. Different letters indicate significant differences of means by pairwise contrasts using a sequential Bonferroni correction.
*Robust estimation used to handle violations of ANOVA assumptions.

Homogeneity of data variances was tested by Levene's test, based on the median, and the normality of residuals was examined using the Shapiro–Wilk test. For three parameters, outliers were detected and winsorized by the closest value (soil $\delta^{15}N$, $\delta^{15}N$ of unlabeled vegetative plant parts, and N concentration of generative unlabeled plant parts). A mixed model with plots as subject, nested in parts or depth, with crops and parts as fixed factors and their interactions were used, followed by a pairwise comparison of crops and interactions using the sequential Bonferroni correction. The different %Ndfa values estimated for lablab using the dilution method with maize or finger millet as reference plant and the natural abundance method were analyzed with a Welch test followed by Games–Howell post hoc tests in the case of inhomogeneous variances. All statistics were conducted using SPSS Statistics Version 28.0.1.0 (IBM Statistics, IBM Corp., Armonk, NY, USA).

3 | RESULTS

The labeling of the soil N pool using 10 at% ^{15}N-enriched N fertilizer given in two split applications during the cultivation period resulted in $\delta^{15}N$ values of 2894‰ for maize, 3586‰ for finger millet, and 1301‰ for lablab ($n = 2$ per crop, CV% = 43%) at 63 DAS whereby unlabeled control plants had respective $\delta^{15}N$ values of 1.4‰, 0.8‰, and 1.7‰. At the end of the experiment, 95 DAS, labeling was strongest in finger millet being 2.6–3.4 times higher in its $\delta^{15}N$ than lablab, followed by maize being 1.7–2.3 times higher than lablab (Table 1). Although maize and finger millet had 17% higher $\delta^{15}N$ in the vegetative biomass compared with the generative biomass, it was slightly lower in lablab. The natural abundance of ^{15}N in unlabeled control plants was highest in maize followed by finger millet and lablab, which had 68%–28% lower $\delta^{15}N$ in the vegetative and generative aboveground biomass, whereby these differences were not significant. At the end of the experiment, the $\delta^{15}N$ of labeled soils at 0–10 cm depth was still 16–22 times higher than in unlabeled soils, whereby it was by 363% and 33% higher in finger millet plots compared with maize and lablab (Table 1). The $\delta^{15}N$ of labeled soil at 10–30 cm depth was 3.5–4.4 times higher than in unlabeled soil, whereby differences between the crops were small.

The aboveground biomass in vegetative and generative parts was highest for maize, with lablab reaching about 40% and finger millet about 20% of this biomass (Table 2). The N concentration of vegetative and generative biomass was highest in lablab, compared with the two grasses maize and millet ($p < 0.001$). The application of ^{15}N fertilizer led to a 2%–50% increase in the N concentration of maize and lablab, whereas in finger millet, it decreased by 8%–17%. Total N uptake in the vegetative and generative aboveground dry matter per hectare was comparable for maize and lablab, whereas finger millet only took up 13%–16% of their N uptake. Similar to total N uptake, NUE of applied ^{15}N fertilizer was lowest in finger millet and 2.2 times higher in lablab and 4 times higher in maize (Table 3). In contrast, Ndff was highest in finger millet with 10% followed by maize (33% less than finger millet) and lablab (68% less than finger millet). As maize was less strongly labeled than finger millet, its use as non-N_2-fixing reference plant was limited. Estimated %Ndfa using maize as reference plant would amount to around 52% for lablab, whereas when finger millet was used as reference, plant %Ndfa reached 68% for lablab and 33% for maize. In contrast, using the natural abundance of ^{15}N for %Ndfa estimation, maize had the highest $^{15}N/^{14}N$ ratio resulting in about 60% Ndfa for lablab and 45% Ndfa for finger millet. The different methods indicated an N_2 fixation of 41–53 kg N ha^{-1} in lablab (Figure 3). Using the natural abundance method resulted in an atmospheric N_2 uptake by millet of 5 kg ha^{-1}.

TABLE 2 Results of the ^{15}N tracer experiment for maize, finger millet, and lablab conducted at the GKVK Campus of the University of Agricultural Sciences Bangalore, Bengaluru, South-India.

		N concentration		Total *N*
	Aboveground dry matter (kg ha^{-1})	Unlabeled control (%)	Labeled crops (%)	Uptake (kg ha^{-1})
Vegetative				
Maize	4545 a	0.80 b	0.88 b	40.3 a
Finger millet	852± c	0.94 b	0.78 b	6.5 b
Lablab	1727 b	2.01 a	2.50 a	42.7 a
Generative				
Maize	1791 a	1.00 b	1.50 b	26.8 a
Finger millet	383 c	1.28 b	1.18 c	4.4 b
Lablab	745 b	4.47 a	4.57 a	34.3 a
PCV%	23.1	14.3	9.4	23.0
p-Value				
Crop	**<0.001***	**<0.001**	**<0.001**	**<0.001**[1]
t	**<0.001**	**<0.001**	**<0.001**	**0.005**
C × P	**<0.001**	**<0.001**	**<0.001**	0.002

Note: Data show average plant biomass per ha, *N* concentration, and N uptake of crops with their mean coefficient of variation (CV%) and *p*-values of a mixed model with plot as subject, nested in depth (for soil) and parts (vegetative and generative for crops) with crops, depth/parts, and their interaction as fixed factors. Different letters indicate significant differences of means by pairwise contrasts using a sequential Bonferroni correction.
*Robust estimation used to handle violations of ANOVA assumptions.

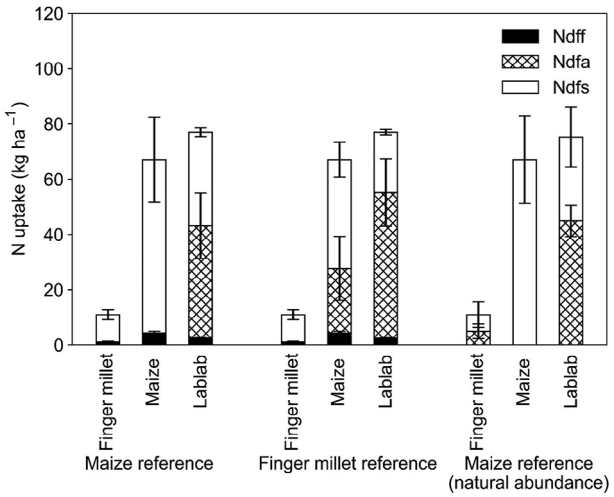

FIGURE 3 N uptake in kg ha^{-1} fractionated in N derived from fertilizer (Ndff), N derived from atmosphere (Ndfa), and N derived from soil (Ndfs) for maize, finger millet, and lablab calculated for the dilution method with different reference plants (maize or finger millet) and based on natural abundance with maize as reference plant in an irrigated field experiment at the GKVK Campus of the University of Agricultural Sciences Bangalore (UASB), Bengaluru, South-India.

4 | DISCUSSION

4.1 | ^{15}N labeling of soil and crops

The data show that the labeling of soils and plants was successful, and the plants did not deplete the pool of supplied ^{15}N. At the end of the experiment, the amount of ^{15}N-labeling remained 22 times higher than natural abundance for finger millet, and ≈16.5 times higher for maize and lablab in the top soil layer, and 3.5–4.4 times higher in the lower soil layer. For finger millet, $\delta^{15}N$ was higher than in lablab and maize soil likely reflecting the lower relative share of plant biomass N taken up from soil than in finger millet. The latter recovered the highest percentage of ^{15}N of total N uptake, but the absolute quantity was lower due to the low biomass production. The labeling was strongest in finger millet, followed by maize and lablab, indicating that maize might have taken up some N from associative N_2-fixation (Bloch et al., 2020; Brusamasello-Santos et al., 2017; Montañez et al., 2009) or from deeper soil levels, leading to stronger dilution of the ^{15}N (Montañez et al., 2009). The strong decrease of $\delta^{15}N$ in finger millet and maize from 65 DAS to 95 DAS is as indication that the plants were able to access unlabeled N-sources, probably from lower soil horizons and that during their final growth a dilution of the $^{14}N/^{15}N$ ratio occurred. However, the natural abundance of ^{15}N was numerically higher in maize than in finger millet and lablab with partly doubled $\delta^{15}N$ values. Natural abundances of crops using N from N_2-fixation have lower $\delta^{15}N$ due to a lower $^{15}N/^{14}N$ ratio in atmospheric N_2 (Högberg, 1997). The natural abundance data rather indicate some associative N_2-fixation by finger millet, for which some varieties are known to engage in interactions with associative N_2-fixing bacteria especially *Azospirillum* (Hafner et al., 1993; Ramakrishnan & Bhuvaneswari, 2014). The $\delta^{15}N$ values of finger millet are similar to those of the diazotrophic lablab, although at 63 DAS when the preliminary leaf harvest occurred, $\delta^{15}N$ in unlabeled leaves tended to be higher for finger millet than for maize. However, variance was high and number of replicates too small ($n = 2$) to verify statistical significance of these trends.

When plant-specific biomass production and N content in harvested aboveground dry matter are taken into account, the N uptake in maize was 6.1 times higher than in finger millet and even 7 times higher in lablab. Although maize took up around 30% of the applied ^{15}N, lablab only utilized 16% and finger millet only 7%. For lablab, this result was expected as it can access unlabeled N from N_2-fixation, and similar values were reported for different varieties of lablab (Gupta et al., 2012). This leads to a dilution of the ^{15}N taken up from the labeled fertilizer, which can be used to calculate the contribution of fertilizer and of atmospheric N_2 (Fuertes-Mendizábal et al., 2018; He et al., 2009; Montañez et al., 2009). For lablab, N_2 fixation was estimated at 51% Ndfa, when maize was used as reference crop based on the dilution method. However, as stated above, finger millet had a stronger labeling questioning our assumption that maize was a good reference crop, because its labeling seemed to be diluted from an unlabeled N source. The selection of a proper reference crop is crucial but also very challenging in tracer experiments, as the reference crop and the

TABLE 3 Total N uptake in the aboveground dry matter by crops, N use efficiency in % of applied fertilizer (NUE), N derived from fertilizer (Ndff), and N derived from atmosphere (Ndfa) with their coefficient of variation (CV%) of a ^{15}N tracer experiment for maize, millet, and lablab conducted at the GKVK Campus of the University of Agricultural Sciences Bangalore, Bengaluru, South-India.

	Total *N* uptake (kg ha^{-1})	NUE (%)	Ndff (% of total *N*)	Ndfa (maize REF)	Ndfa (millet REF)	Ndfa (maize REF nat. abun.)
Maize	67.1 a	30.2 a	6.9 b	REF	33.2	REF
Finger millet	11.0 b	7.6 c	10.3 a	NA	REF	45.1 ± 13.99
Lablab	77.0 a	16.8 b	3.3 c	51.9 B	67.9 A	59.6* AB
CV%	19.6	14.9	14.92	28.3		
p-Value	**<0.001**	**<0.001**^{W}	**<0.001**	**0.019**		

Note: *p*-Values are the results of a mixed model Analysis of Variance (ANOVA) with plot as subject and crops as fixed factors or when indicated by Welch tests. Different letters indicate significant differences of means as per Games–Howell post hoc tests.
Abbreviations: NA, no valid values available, REF, reference crop; SEM, average standard error of the mean; W, Welch test.
*$n = 3$.

N_2-fixing crop should have a similar pattern of soil N uptake, because ^{15}N-enrichment of soil inorganic N changes over time (Bremer et al., 1993). To improve the accuracy of N_2-fixation estimates, the use of several reference crops or the comparisons with the natural abundance method were proposed by the same authors. In the current experiment, the use of other reference crops or repeated data collection on the same micro-plots over time was unfeasible due to the fixed crop rotation of the long-term crop rotation experiment. With finger millet as reference plant, estimated %Ndfa of lablab amounted to 68%, whereas the natural abundance method with maize as reference plant resulted in 60% Ndfa. Lablab fixed 41–53 kg N ha^{-1}, which would be substantially higher than the 20 kg ha^{-1} N_2-fixation reported in previous studies (Rocherster et al., 1998). The similar soil labeling in maize and lablab plots at the end of the experiment and the similarity of total N uptake in the aboveground dry matter of both crops indicate that maize was an appropriate reference crop in our study. For maize, estimated %Ndfa using finger millet as reference crop would amount to about 33%. There is evidence that maize can be involved in rhizosphere and endophytic associations with N_2-fixing bacteria (Montañez et al., 2009). However, the natural abundance of unlabeled maize was numerically higher than that of finger millet, which indicates that associative N_2-fixation may have contributed to total N-uptake only to a very minor degree if at all. For finger millet instead, maize might not be the best reference crop, as it might take up N from deeper, less strongly labeled soil layers not accessible to finger millet, leading to a dilution in maize but not in finger millet. With the natural abundance method, these different root systems are less problematic as the ^{15}N concentration in the soil N pool is more homogeneous, although Watanabe et al. (1987) reported that the $\delta^{15}N$ value of NH_4^+ increased with soil depth and might have caused differences in $\delta^{15}N$ of different wetland rice genotypes. Although the %Ndfa values calculated for lablab were in a similar range for the dilution method and the natural abundance method, associative N_2-fixation is more difficult to quantify due to the smaller contribution of atmospheric N to total N uptake and the choice of a suitable reference crop is difficult. The %Ndfa for finger millet calculated using the natural abundance method with maize as a reference crop yielded 45%, which translates to about 5 kg N ha^{-1} due to the very low total N uptake in the aboveground dry matter by finger millet. This contribution of atmospheric N_2 to the N nutrition of finger millet might explain the unexpectedly higher yields of finger millet, but most likely, it is not relevant for maize as a follow-up crop.

5 | CONCLUSIONS

It is surprising that the studied crops did not utilize the readily available ^{15}N from urea and ammonium sulfate to a larger extent. Although a maximum finger millet NUE of 7% has previously been described in the literature, we expected a maize NUE of around 50% rather than the 33% measured in our study.

The data showed that lablab as a symbiotic N_2-fixing plant derived between 51% and 68% of its aboveground N from the atmosphere, which amounted to 41–53 kg ha^{-1}. While characterizing the subtropical and deeply weathered soil at the study site as poorly nitrogenic, substantial diazotrophic N_2-fixation has clearly been shown here for lablab. Differences in ^{15}N-labeling between finger millet and maize likely reflect different rooting depths and different NUEs. The results of the natural abundance method indicate that maize did not fix N_2, but rather ^{15}N dilution has occurred from another N source. For finger millet, only the natural abundance method was useful in our study. It resulted in %Ndfa of 45% of total N uptake, equivalent to 5 kg N ha^{-1}.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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