Plant Soil
https://doi.org/10.1007/s11104-018-3897-x

REGULAR ARTICLE

Atmospheric nitrogen fixation by gliricidia trees (*Gliricidia sepium* (Jacq.) Kunth ex Walp.) intercropped with cocoa (*Theobroma cacao* L.)

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Received: 20 December 2017 / Accepted: 9 November 2018

Abstract
Aims The objective was to estimate the fixation of atmospheric nitrogen (Ndfa) by legume (*Gliricidia sepium*) trees for the benefit of cocoa (*Theobroma cacao*) trees in agroforestry systems.
Methods Four sites where cocoa and gliricidia were intercropped and one site where gliricidia, cocoa, and orange trees were grown as separate stands were selected in Ghana. Ndfa in gliricidia and cocoa leaves (from all sites) and in shoot axes (in one site only) was assessed by the ^{15}N natural abundance technique. Cocoa trees distant (> 10 m) from the closest gliricidia were used as reference plants.
Results With few exceptions, leaves of gliricidia and cocoa trees growing in proximity had similar $\delta^{15}N$, whereas the foliar $\delta^{15}N$ value of gliricidia was lower than that of distant cocoa trees. The Ndfa in gliricidia leaves ranged from 22 to 50% of total leaf N and was 48% in the shoot axis. Root nodules, found only after the wet season, always showed the inner red color indicating effective N_2 fixation and the occurrence of *Rhizobium tropici* and *Rhizobium etli*. The annually produced shoots of gliricidia, theoretically suitable to become green manure after pruning, contained 31.4 to 38.0 kg N ha^{-1} derived from the atmosphere.
Conclusions Gliricidia sepium trees are able to take advantage of the association with rhizobial symbionts to fulfill, at least in part, the N needs of their rapidly growing shoots. In mixed-stand agroforestry systems, with intercropped gliricidia and cocoa trees, the amount of N derived from the atmosphere that could enter the soil if the pruned shoots of gliricidia trees are used as green manure could diminish the need for N fertilizers for cocoa trees.

Keywords Green manure · Legume trees · ^{15}N natural abundance · Nitrogen fixation · Rhizobia · Shoot pruning

Responsible Editor: Euan K. James.

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Introduction

Ghana is the second largest producer of cocoa in the world and over 6 million Ghanaians depend on the cocoa sector for their livelihood (Kolavalli and Vigneri 2011). In recent years, cocoa farmers in Ghana have been confronted with the challenge of yield decline and, in many growing regions, the average yield is 0.45 Mg cocoa beans ha^{-1}, much lower than the potential achievable yields of 0.8–1.0 Mg ha^{-1} (Baffoe-Asare 2013). In addition to drought, one important cause of such yield decline is the depletion of soil nitrogen (N) due to continuous cropping with little or no N replenishment

Published online: 01 December 2018

(Baah et al. 2011; Aneani et al. 2011). Soil N deficiency, causing stunted cocoa growth, fruit abortion, and poor pod yields (Tscharntke et al. 2011) could be prevented by the supply of mineral N fertilizers, but these are often unaffordable for smallholder farmers, who represent the majority of cocoa farmers in Ghana (Opoku-Ameyaw et al. 2012; Nunoo et al. 2014). Moreover, many remote regions of Ghana are not easily accessible and recent estimates indicate that only 20% of the 1.6 million hectares of cocoa farms in Ghana receive mineral fertilizers (IFDC 2012).

N_2-fixing legume trees are common in tropical agroforestry systems and, if properly managed, they may provide an alternative to N fertilizers as a means for enhancing soil N availability to the benefit of the non-legume plants. In their review, Nygren et al. (2012) listed more than 15 tree species belonging to Fabaceae, present in humid or sub-humid agroforestry systems, whose N derived from atmosphere varied from 28 to 92%, with ranges for each species depending on the organ considered, the microbial strain, the tree age, the pruning frequency, the sampling time, and the reference species. Some of these trees, like *Inga edulis* and *Gliricidia sepium*, also provide the shade required by the cocoa tree. Nitrogen deriving from biological fixation (BN) is a cheap, practically unlimited, and environmentally friendly N source. *Gliricidia sepium* (Jacq. Kunth ex Walp.), hereafter gliricidia, is capable of forming a symbiotic association with N_2-fixing rhizobia (Kinkema et al. 2006) and produces high-quality biomass for use as green manure (Subramanian et al. 2005).

To account for the potential of gliricidia in introducing N from BNF, ^{15}N isotopic techniques are apparently the most recommended for field studies (Boddey et al. 2000). The acetylene reduction assay (ARA), although useful to assess the nitrogenase activity, is unsuitable to quantify the N fixed under field conditions (Chalk et al. 2017). A few studies have attempted to estimate N fixation by the ureide abundance in xylem sap (Unkovich et al. 2008), but the required relationship between %Ndfa and ureide abundance for gliricidia was not developed, yet. The ^{15}N enrichment and the ^{15}N dilution technique are difficult to apply under field conditions in view of problems related to the dilution of the labeled fertilizer N, with unlabeled soil mineral N that may lead to an unequal ^{15}N enrichment in space and time (Unkovich et al. 2008). The ^{15}N natural abundance method ($^{15}NNAM$) is the best option for estimating N_2 fixation in tree plantation and agroforestry systems as the soil available N is naturally enriched with ^{15}N, allowing the quantification of N_2-fixation integrated over one season or even over longer periods (Boddey et al. 2000; Unkovich et al. 2008; Khamzina et al. 2009).

The $^{15}NNAM$ is based on the difference in $\delta^{15}N$ values between two N sources (soil mineral N and atmospheric N_2) for the plant (Unkovich et al. 2008). The $^{15}NNAM$ requires one or more non-N-fixing reference plants that provide an integrated determination of the ^{15}N of the soil mineral N available for plant growth over the duration of the study (Boddey et al. 2000). Such reference plants should have roots with similar vertical distribution of the N_2-fixer plant and be far enough from the latter so that the residues of one type of plant do not affect the ^{15}N abundance of the soil available N.

In this study, we have (i) molecularly characterized the bacterial communities of gliricidia root nodules, and assessed (ii) the fraction of N derived from the atmosphere present in shoots of gliricidia trees intercropped with cocoa, and (iii) the potential transfer of atmospheric N to the neighboring cocoa trees through the pruning of gliricidia shoots.

Methodology

Experimental sites

The experiment was carried out in two cocoa-growing regions of Ghana (West Africa). Three cocoa plantations (referred to as sites 1, 2, and 3), where gliricidia trees were present as an intercropped species, were sampled, both at the end of the dry (January 2015) and of the wet (September 2015) seasons. A fourth and a fifth site (site 4, 5) were sampled only at the end of the wet season. At sites 1–4, cocoa trees were planted with 3 × 3 m spacing while gliricidia trees were dispersed at variable distances from cocoa trees, from less than 5 to more than 10 m. At site 5, gliricidia, cocoa, and orange trees were not intercropped but grown as separate stands in neighboring plots with a spacing of 9 m × 9 m (gliricidia) and 3 m × 3 m (orange and cocoa).

The size of the plots ranged from 0.20 to 0.85 ha (Table 1). Sites slightly differed in type of management and intercropping characteristics (Table 1). All sites were located at an altitude between 200 and 250 m a.s.l. Sites 1, 2, and 5 are located in the Ashanti region and are part of the experimental farm of the Faculty of Renewable Natural Resource, Kwame

Table 1 Some management characteristics of the selected sites

Characteristics	Site 1	Site 2	Site 3	Site 4	Site 5
Age of the cocoa plantation (years) and plot size (ha; in brackets)	8 (0.25)	9 (0.85)	15 (0.40)	6 (0.35)	11 (0.20–0.40 depending on the crop)
Type of intercropping	Gliricidia intercropped in rows with cocoa (25% gliricidia and 65% cocoa).	Gliricidia is sparingly intercropped with cocoa (15% gliricidia and 70% cocoa).	Gliricidia is sparingly intercropped with cocoa (68% cocoa and 17% gliricidia).	Gliricidia is sparingly intercropped with cocoa (18% gliricidia and 62% cocoa).	Pure gliricidia, cocoa and orange stands.
Gliricidia cultural practice	Top pruning in December, with pruned shoots left on the soil where they fell.	Lateral pruning in December, with pruned shoots left on the soil where they fell.	Top and lateral pruning in December. Part of the biomass used as forage and part left on the soil where they fell.	Top pruning in December, with pruned shoots left on the soil where they fell.	Top and lateral pruning in December, with pruned shoots left on the soil where they fell.
Nutrient management	No external nutrient supply.	Inorganic N fertilizers, mainly Ca nitrate (approximately 12 kg N ha^{-1}) in the previous 5 years.	Ca nitrate yearly applied (approximately. 20 kg N/ha) since establishment.	No external nutrient supply.	No external nutrient supply.
Average cocoa yield (beans)	n.a.	175 kg ha^{-1}	188 kg ha^{-1}	n.a.	n.a.

n.a. not available

Table 2 Main climate characteristics, soil type, and geographic coordinates of study sites in the Ashanti and Eastern regions of Ghana

Region	Average annual rainfall (mm)	Average yearly temperature (°C)	Soil type (FAO classification)	Geographical coordinates
Ashanti	1375	26.6	Ferric Acrisol	Site 1: Latitude 6° 40′ N; Longitude 1° 34′ W
				Site 2: Latitude 6° 39′ N; Longitude 1° 33′ W
				Site 5: Latitude 6° 40′ N; Longitude 1° 34′ W
Eastern	1650	25.5	Rhodic Ferrasol	Site 3: Latitude 6° 17′ N; Longitude 0° 27′ W
				Site 4: Latitude 6° 17′ N; Longitude 0° 27′ W

Nkrumah University of Science and Technology (KNUST) in Kumasi (Table 2). Sites 3 and 4 are located in the eastern region and within the experimental farm of the Bunso Cocoa College in Bunso. Both regions form part of the semi-deciduous forest zone of Ghana (Aneani et al. 2011) and are characterized by a bimodal distribution of rainfall, with a main wet season from March to July and a minor wet season that starts in late August and lasts until November (Anim-Kwapong 2003; Partey et al. 2011) (Table 2). The soils in all sites have sandy loam texture. Soil N concentration ranged from 0.08 to 0.14% (Table 3). Gliricidia trees are normally pruned in December and the pruned shoots left beneath the trees as green mulch. The horizontal spread (maximum diameter of the soil projection of the canopy) of the gliricidia trees was measured at the end of the wet season in 2018 in sites 2, 3, and 4 on four randomly chosen trees per site and averaged 10.0 m (standard deviation, s.d. = 2.2 m) tree^{-1}, a value in line with literature data (Elevitch and Francis 2006).

Nitrogen derived from the atmosphere (Ndfa)

To assess the percentage of N derived from the atmosphere (% Ndfa), the ^{15}N natural abundance method (^{15}NNAM) was adopted. The technique is based on the comparison of the delta (δ) ^{15}N of a plant that develops symbiosis with N_2-fixing microorganisms with one or more reference plants, whose N derives entirely from soil N forms. The δ^{15}N of the atmospheric N_2 is defined as 0; the isotopic composition of other N sources is normalized to the atmospheric composition (Shearer and Kohl 1986). Within each site, three subplots (about 500–800 m^2 each) were randomly selected, one gliricidia tree and two cocoa trees, one close (cocoa C), and the other distant (cocoa F) to the closest gliricidia tree were used for sampling. The means and standard deviations of the distance of selected cocoa C and cocoa F from the closest gliricidia tree were 5.48 ± 0.35 and 13.04 ± 0.77 m, respectively. Sixty to eighty young, fully-expanded leaves were sampled from current year shoots from each sampled tree. At site 5, three

Table 3 Main soil chemical characteristics and δ^{15}N values from 0 to 45 cm

	Site Unit	1	2	3	4	5
pH ($CaCl_2$)		5.2	5	5.5	5.8	5.8
Organic C	g kg^{-1}	13	16.5	11.9	10.3	8.9
Total N	g kg^{-1}	0.8	1.1	0.8	0.9	1.0
δ^{15}N of total soil N	‰	7.2 ± 0.4	7.5 ± 0.2	7.1 ± 0.2	7.0 ± 0.3	7.0 ± 0.1
Available P	mg kg^{-1}	6	4	4	4	3
Extractable K	mg kg^{-1}	21	14	22	25	25
Extractable Mg	mg kg^{-1}	7	6	7	8	7

sub-plots were selected within separate stands of gliricidia, cocoa, and orange trees. Leaves of orange and shoot axes of gliricidia, in addition to cocoa and gliricidia leaves, were collected at site 5.

In September 2015, three soil samples from each sub-plot were collected from the 0–30-cm upper layer (Hartemink 2005), at least 5 m from the closest gliricidia tree (sites 1–4) and in the cocoa separate stand present at site 5. Soil samples were analyzed for chemical and physical properties as well as for $\delta^{15}N$, to check one of the requirements for applying the ^{15}NNAM. Soil pH was determined in 0.01 M $CaCl_2$ (1:2.5). Total N, $\delta^{15}N$, and organic C (after soil acidification using 6 M hydrochloric acid) were measured at the Isotope Laboratory of the Free University of Bozen-Bolzano, Italy, using an elemental analyzer coupled with a continuous flow isotopic mass spectrometer (Flash 2000 and Delta V, Thermo Scientific Instrument). The extractable magnesium (Mg) was analyzed at the agricultural chemistry laboratory of the Research Centre for Agriculture and Forestry of Laimburg according to Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten (Bassler et al. 1991). The "plant-available" potassium (K) and phosphorous (P) were extracted in calcium-acetate-lactate (CAL solution) according to the Austrian standards (ÖNORM L 1087 2012).

The sampled leaves were cleaned from dust and oven-dried at 60 °C for 72 h. The dried material was milled into a homogenous powder using a laboratory ball-and-capsule vibrating mill. About 2.0–2.5 mg of milled material was encapsulated in tin and analyzed for their percentage nitrogen (% N) and ^{15}N (using the same instrument described above).

The percentage of nitrogen derived from the atmosphere (% Ndfa) in the gliricidia plant was estimated according to Shearer and Kohl (1986) and Boddey et al. (2000) using the formula:

$$Ndfa = \frac{\delta^{15}N_{reference} - \delta^{15}N_{gliricidia}}{\delta^{15}N_{reference} - B} \cdot 100$$

where $\delta^{15}N$ reference is the $\delta^{15}N$ of leaves from the non-N-fixing plant (cocoa for sites 1–4 and both cocoa and orange for site 5) and $\delta^{15}N$ gliricidia is the $\delta^{15}N$ value of gliricidia leaves or shoot axes. *Theobroma cacao* proved to be suitable as a reference plant in a study carried out by Nygren and Leblanc (2009), giving similar results of Ndfa as three other non-legume reference species. The B value represents the $\delta^{15}N$ value of gliricidia when N_2 is the sole N source for the tree. In our calculations, we used four B values that had been experimentally determined for *Gliricidia sepium* leaves or shoots, − 0.45‰ (Anhar 2005), − 1.45‰ (Ladha et al. 1993), − 1.11‰ (Hairiah et al. 2000), and − 2.07‰ (Nygren et al. 2000).

Assessment of growth and nitrogen uptake by gliricidia

To estimate the amount of nitrogen fixed by gliricidia trees, the biomass of shoots grown in 1 year and their N content were determined. This part of the study was carried out at site 1 only. In January 2015, six 5-year-old gliricidia trees, whose circumference at 20 cm from the ground varied from 17.9 to 22.7 cm, were randomly selected. The average number of branches per each tree was 13 (range 9–15). Five branches per tree were tagged and within each branch, the terminal point of previous season growth of each shoot was identified and marked. The average number of shoots per branch was 10 (range 7–14) and the number of leaves per shoot was 19 (range 17–27). In January 2016, the tagged branches were cut and the new growth separated into leaves and shoot axes, which were oven-dried at 70 °C for 72 h and weighed. The average dry weight (DW) of leaves and shoots present on the sampled branches was multiplied by the total number of branches per tree to estimate the total biomass of leaves and shoot axes per tree. Samples were analyzed for total nitrogen concentration and the total amount of N in leaves and shoot axes was calculated.

Analysis of root nodules and nodule bacteria

Gliricidia-nodulated root samples were collected from the study sites 1–3 at the end of the dry (January 2016) and wet (September 2016) season and only at the end of the wet season at site 4. Three gliricidia trees per site were sampled, with the exception of site 3 where only two trees were sampled. Under each tree, four holes (40 × 40 cm with 15 cm depth) were dug 45–60 cm distant from the trunk.

After the dry season, we did not find root nodules in any of the sites, whereas after the wet season, nodules were always present. When present, root nodules were visible with the naked eye. Roots collected from each hole were used to prepare a composite, representative root sample for each tree (10–20 g). Roots were cleaned from soil particles, left under shade for 6–12 h to remove excess water (only after the September sampling),

placed in paper envelopes and wrapped in a polyethylene bag, transported to Italy in cooling flasks to avoid desiccation, and processed within 48 h.

To assess root nodules, root samples from sites 1–4 were washed with tap water and wiped. Sub-samples of about 1.8 g each were collected and analyzed under a dissecting microscope (Wild, Leica, Milan, Italy) to quantify the number of nodules.

A culture-independent method, PCR-DGGE, was used to identify the bacteria occurring in the nodules. Ten nodules were collected for each sample, re-hydrated in sterile distilled water, and externally sterilized by immersion in ethanol (70% *v*/*v*) for 2 min and then in sodium hypochlorite (3% *v*/*v*) for 4 min, followed by two rinses of 30 min in sterile water. Sterilized nodules were crushed with sterile plastic pestles in 150 μl of the "PowerSoil™ DNA Isolation Kit" extraction buffer (Mo-Bio Laboratories Solana beach, CA, USA). DNA was isolated according to the manufacturer's instructions. For analysis of bacterial communities, the amplification of the variable region V3–V5 of the 16S rRNA gene was carried out using the primers 341F and 907R (Yu and Morrison 2004), as reported in Agnolucci et al. (2015).

For DGGE separation, the amplicons were analyzed using the DCode™ Universal Mutation Detection System BIORAD and a gel with a urea-formamide denaturing gradient ranging between 35 and 65%. A composite mix of bacterial 16S rRNA gene fragments from *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) IMA N29., *Bacillus* sp. IMA CH19, *Streptomyces* spp. IMA W77, and *Mesorhizobium ciceri* IMA M620 were added as reference DGGE marker (M). Gels were run and visualized as described in Agnolucci et al. (2013). DGGE fragments were cut out from the gels for sequencing (Agnolucci et al. 2015). Sequences were analyzed using BLAST on the NCBI web and submitted to the European Nucleotide Archive, under the accession numbers from LT797814 to LT797824.

Statistical analysis

The effects of plant type (gliricidia, cocoa C, and cocoa F) and sampling season (after dry and wet periods) on $\delta^{15}N$ and total N concentration of leaves sampled from sites 1–3 were verified using an ANOVA after having checked the assumptions of normal distribution and the homogeneity of variance. A one-way ANOVA was similarly used to assess differences in total N concentration and $\delta^{15}N$ among gliricidia, cocoa C, and cocoa F at site 4 as well as among gliricidia organs, cocoa, and orange at site 5. The probability level of 5% was considered for the statistical significance; though where appropriate, *p* values less than 1% are stated. Mean separation was carried out using the Tukey's Honestly Significant Difference (HSD) procedure at 5% level of probability. Statistical analysis was performed using StatGraphic Centurion XV (StatPoint, Inc.). Where shown, data are averages ± standard deviation (s.d.) of the three replicates per site and season.

Results

Nitrogen derived from the atmosphere

Nitrogen concentrations were significantly ($p < 0.01$) higher in gliricidia than in cocoa leaves, regardless of site, season, or distance between cocoa and gliricidia (Table 4, Fig. 1). With the exception of gliricidia and cocoa F at site 2, both cocoa and gliricidia leaves had higher N concentration after the dry than after the wet season (Table 4). At site 5, gliricidia leaves also had higher N concentrations as compared to orange leaves and gliricidia shoot axes (Fig. 1).

The $\delta^{15}N$ of total soil N in the five sites ranged from 7.0 to 7.5‰ (Table 3). The effects of season and plant type on leaf $\delta^{15}N$ were different across sites (Table 5). With the exceptions of gliricidia and cocoa C at site 1, leaf $\delta^{15}N$ was always higher after the wet than after the dry season (Table 5). The $\delta^{15}N$ value of gliricidia leaves

Table 4 N concentration (% N DW) of cocoa and gliricidia leaves after the dry and the wet seasons

Site	Season	Gliricidia	Cocoa	
			Close	Far
1	Dry	4.11 aA	2.35 bA	2.35 bA
	Wet	3.70 aA	1.58 bB	1.55 bB
2	Dry	3.16 aA	2.31 bA	2.05 bA
	Wet	3.71 aA	1.82 bB	2.03 bA
3	Dry	3.91 aA	2.36 bA	2.47 bA
	Wet	3.59 aA	1.58 bB	1.63 bB
4	Wet	3.67 a	1.82 b	1.99 b

Different lowercase letters in rows indicate significant ($p < 0.05$) differences between type of plant (species and distance) within each site and season; different uppercase letters in columns indicate significant ($p < 0.05$) differences between seasons within each site

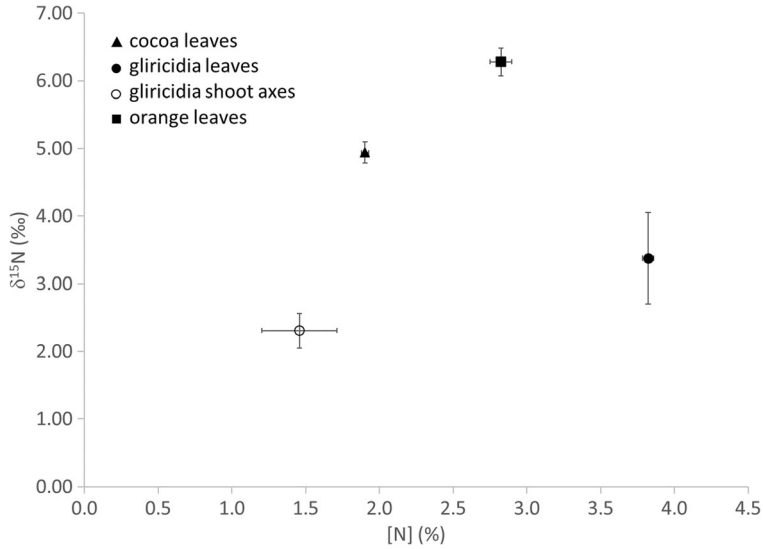

Fig. 1 N concentration (% DW) and $\delta^{15}N$ of selected gliricidia organs, cocoa leaves, and orange leaves sampled from separate stands (site 5). Bars indicate standard deviations

was lower than that of cocoa F for both sampling periods at site 2 and at sites 1, 4, and 5 after the wet season (Table 5, Fig. 1). With the exception of site 3 and the dry season sampling at site 1, leaves from cocoa C had lower $\delta^{15}N$ than cocoa F, and were generally similar to gliricidia. Gliricidia leaves and shoot axes had similar $\delta^{15}N$ at site 5, and both had lower $\delta^{15}N$ than orange leaves (Fig. 1).

% Ndfa by gliricidia could be assessed after the wet season at sites 1, 4, and 5, for both sampling periods at site 2, but neither sampling period for site 3, nor the dry period at site 1, when the $\delta^{15}N$ of gliricidia and the reference plant did not significantly differ. The average of estimates of % Ndfa present in gliricidia leaves using four different B values varied from 22% in the sampling carried out after the wet season at site 2 to 50% in the sampling carried out after the wet season in site 4 (Fig. 2). At site 5, the choice of cocoa or of orange as reference trees affected the estimates of % Ndfa of gliricidia leaves, 25% and 39%, respectively. The use of four B values from the literature resulted in four estimates of % Ndfa of gliricidia leaves, whose coefficient of variation was 10% on average (Fig. 2). At site 5, shoot axes averaged 48 ± 5% of N from the atmosphere (data not reported in the figure).

Between January 2014 and January 2015, each gliricidia tree increased its shoot biomass by approximately 38 kg on average (DW). The shoot axis:leaf ratio was 7:1. Leaves had much higher N concentration than shoot axes, but due to their lower biomass, their N content was approximately 30% of the total shoot N (Table 6).

Table 5 $\delta^{15}N$ (‰) of gliricidia and cocoa leaves after the dry and wet seasons

Site		Gliricidia	Cocoa	
			Close	Far
1	Dry	2.7 aA	2.9 aA	3.3 aB
	Wet	3.5 bA	2.8 cA	5.3 aA
2	Dry	2.2 bA	2.6 bB	3.9 aA
	Wet	3.3 aA	3.2a A	4.6 aA
3	Dry	3.8 aA	2.5 aA	3.1 aB
	Wet	3.9 aA	3.8 aA	4.2 aA
4	Wet	3.6 b	4.4 b	8.5 a

Different lowercase letters in rows indicate significant ($p < 0.05$) differences between type of plant (species and distance) within each site and season; different uppercase letters in columns indicate significant ($p < 0.05$) differences between seasons within each site

Analysis of root nodules and nodule bacteria

No nodules were found after the dry season, while nodules were present after the wet season and their density was assessed (Table 7). All crushed nodules showed an intense red color due to the presence of leghemoglobin, which is a characteristic of effective N_2 fixation.

DGGE analysis revealed the presence of *Rhizobium tropici* and *Rhizobium etli* at all the four sites. In addition, other sequences from the four sites showed 99% similarity with *Rhizobium* sp. strain 6B. Moreover, sequences

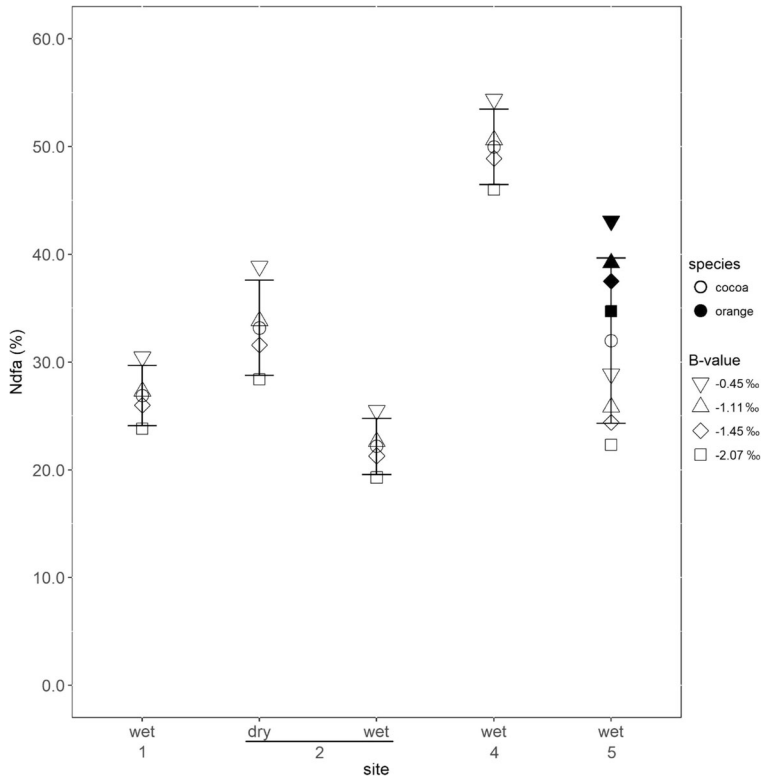

Fig. 2 % Ndfa in gliricidia leaves collected after the dry and the rainy season calculated using four B values retrieved from the literature (see legend). Reference plants were cocoa in sites 1, 2, and 4 (white symbols) and both cocoa (white symbols) and orange (black symbols) trees in site 5. The white empty circle indicates the average of each set of data and the vertical bars indicate the standard deviation

affiliated to *Sporosarcina thermotolerans* strain CCNWQLS96 (100% similarity) and to *Planomicrobium chinense* strain RT4 (99% similarity) were retrieved from site 1 (Fig. 3).

Discussion

In this study, we have quantified the N deriving from atmospheric N fixation in *Gliricidia sepium* trees, assessed the occurrence of effective nodules, and then molecularly characterized their microbiota. The number of effective nodules per gram of root ranged from 62 to 92, values that can be hardly compared with literature data as, to our knowledge, no studies have assessed root nodule numbers of legume trees in the field, while most of them have been carried out in vitro or in microcosm experiments, where the whole root systems were harvested and analyzed after short-time experiments, i.e., 3 to 13 weeks (Rosendahl et al. 1989; Tsai et al. 1998). Here, in order to identify the bacterial communities occurring in the nodules, a culture-independent approach, PCR-DGGE, was chosen, as it is able to overcome the problem of underestimation consequential to the constraints of culture media and cultivation conditions, as well as to detect the presence of bacteria in Viable But Not Culturable (VBNC) state (Agnolucci

Table 6 Estimated annual shoot growth (DW = dry weight) of gliricidia trees and its N content. Data refer to the site 1 (mean ± standard deviation)

Trunk diameter (cm)		19.8 ± 3.4
N concentration (% DW)	Leaves	4.19 ± 0.16
	Shoot axes	1.34 ± 0.02
Total biomass kg (DW) tree^{-1}	Leaves	4.48 ± 3.58
	Shoot axes	32.74 ± 9.39
Amount of N (kg tree^{-1})	Leaves	0.19 ± 0.07
	Shoot axes	0.44 ± 0.11

Table 7 Number of nodules per gram of root fresh weight (mean ± standard deviation)

Site	No. nodules g^{-1} root
1	65 ± 33
2	92 ± 50
3	63 ± 25
4	62 ± 16

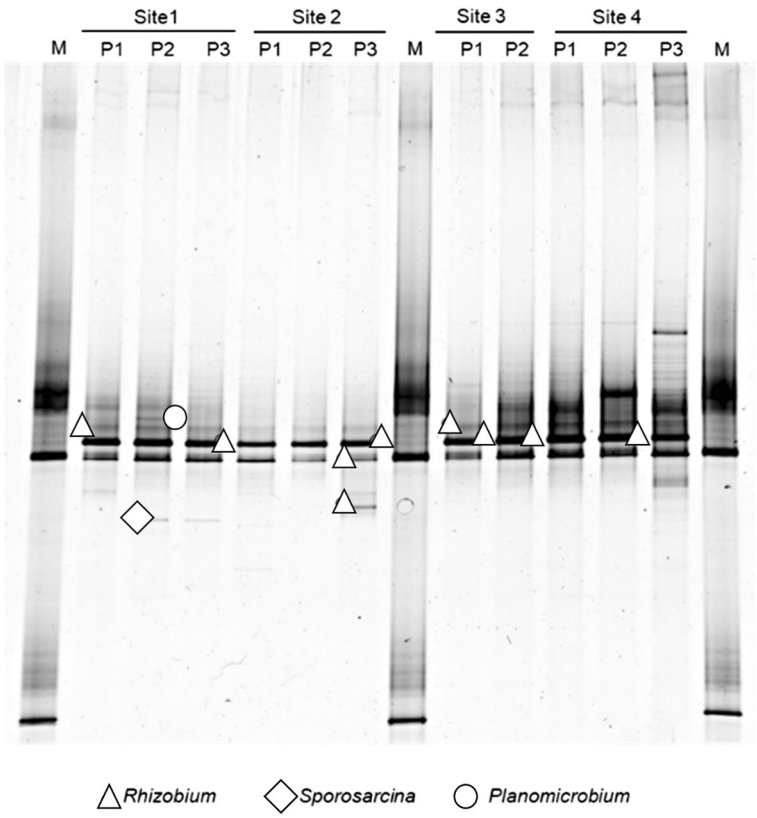

Fig. 3 DGGE analysis of bacterial communities occurring in *Gliricidia sepium* root nodules from sites 1–4. P, replicate plants; M, markers

et al. 2015). The analysis of excised DGGE bands revealed sequences affiliated to *R. tropici* and *R. etli*. *Rhizobium tropici* has been reported as associated with nodules of bean and leguminous trees, including *G. sepium* (Martínez-Romero et al. 1991; Nick et al. 1999; Acosta-Durán and Martínez-Romero 2002), while *R. etli* has been retrieved from a broad number of host species (Hernandez-Lucas et al. 1995). At all four sites, we found one sequence showing 99% similarity with *Rhizobium* sp. strain 6B, which has been described as associated with *Vachellia karroo* in South Africa (Nxumalo et al. 2016).

The %Ndfa in gliricidia shoots varied among the sites: the lowest and the highest values were recorded after the wet season at site 2 (22%) and 4 (50%). Explaining the differences in the %Ndfa in gliricidia organs across sites is beyond the scope of the study, although we might speculate that the age of the trees (Leblanc et al. 2007) and the availability of soil-derived N may account for the differences. It is also known that, at least within a given range, increasing soil phosphorous (P) availability has a positive effect on N fixation rates (Isaac et al. 2011). Soil available P levels in the five sites of the present study are within typical range found in a survey carried out in cocoa plantations in Ghana (Afrifa et al. 2010), but likely lower than the optimal soil P levels for cocoa production. In their review on dinitrogen fixation in agroforestry system, Nygren et al. (2012) averaged data from several published studies and reported Ndfa values of 67% (± 13%) for gliricidia trees, while Apolinário et al. (2016) reported Ndfa of 55% for *G. sepium* grown in the State of Pernambuco, Brazil.

The effect of the sampling period on % Ndfa was only observed at site 2, where slightly higher values were found after the dry than after the wet period (Fig. 2). However, interestingly, we did not find root nodules after the dry season, similarly to what has been reported for other legume trees like *Sesbania sesban* (L.) Merr. and *Leucaena leucocephala* (Lam.) de Wit (Fownes and Anderson 1991; Dirzo et al. 2012). These two results could be regarded as conflicting. It should be considered, however, that root nodule sampling was carried out at the end of the dry period when soil moisture had reached its lowest value, while the $\delta^{15}N$ of the leaves sampled in January likely reflected an average value of dinitrogen

fixation for the entire dry period. In addition, it has been reported that root nodulation ceases following shoot pruning, which in our study was carried out in December, and that the trees remain for several weeks unnodulated after pruning (Nygren and Ramírez 1995; Nygren and Cruz 1998). We cannot exclude some tree-internal N remobilization from leaves developed in the wet period to those growing in the dry period. Our data, therefore, do not contradict the hypothesis that atmospheric N fixation in gliricidia trees is mostly active when water availability in the top soil is not limiting and rapid shoot growth occurs (Nygren and Leblanc 2009).

As stressed by multiple authors (Boddey et al. 2000; Nygren and Leblanc 2009), the estimation of the N contribution by legume trees to the N cycle in agroforestry systems under field conditions is a methodologically-challenging task. The ^{15}N natural abundance techniques provides reliable estimates of biological N fixation when several conditions are met (Shearer and Kohl 1986; Ladha et al. 1993; Gehring and Vlek 2004), including the correct choice of the reference plant, the presence of soil $\delta^{15}N$ higher than 5‰, and the choice of the organs to be sampled. We have not assessed the $\delta^{15}N$ of the mineral N and are aware that some discrimination (2.4‰, Hobbie and Högberg 2012) against ^{15}N occurs during the ammonification and nitrification processes, which explains the depletion of plant N of cocoa trees as compared to total soil N (Table 3). In our study, we have used cocoa trees as reference plants in four sites, and both cocoa and orange on site 5. As a reference plant, *T. cacao* alone gave similar estimates for N_2 fixation of *Inga edulis* Mart. and *G. sepium* as a group of non-N_2-fixing reference species in the study by Nygren and Leblanc (Nygren and Leblanc 2009). Both *T. cacao* and *G. sepium* have quite superficial root systems, mainly concentrated in the upper 0–30-cm soil layer, where also most soil available N is present (Schroth and Zech 1995; Rowe et al. 2001; Nygren et al. 2013). As far as reference plant choice is concerned, if we had used only orange trees—also known for a superficial root system—instead of cocoa trees at site 5, we would have obtained an additional increase in Ndfa by 13 and 11%, for leaves and shoot axes, respectively. We might then speculate that our estimates for the other sites, where only cocoa was used as a reference plant, are likely conservative. The choice of the B value also had an impact on the final assessment of % Ndfa; for instance, using either a B value of − 0.45‰ or of − 2.07‰ would result in % Ndfa estimates differing from a minimum of 6% (site 1) to a maximum of 11% (site 2, dry sampling, Fig. 2).

The fact that we did not find differences in the $\delta^{15}N$ of cocoa and gliricidia at site 3 for both samplings, in spite of the presence of nodules of rhizobia on gliricidia roots, could be due to the regular supply of synthetic N fertilizers in this cocoa plantation, whose $\delta^{15}N$ is similar to that of the atmosphere (Bateman and Kelly 2007).

To estimate the amount of N deriving from the atmosphere that can become available to cocoa trees due to the presence of legume trees, not only are reliable estimates of the % Ndfa needed, but it is also necessary to quantify the amount of total N present in the shoots that can potentially be removed by annual pruning. Such amounts depend on, among others, soil fertility, tree growth potential, and tree density. When gliricidia trees were grown at high tree density in hedgerows (2500 trees ha^{-1}) in Northern Lampung (Sumatra), the shoot biomass that could be pruned annually amounted to more than 4 Mg ha^{-1} (DW) and its N content was equal to 104 kg ha^{-1} (Hairiah et al. 2000), an amount similar to what is reported by Apolinário et al. (2016) for N in annual litter (105–109 kg N ha^{-1}) produced by gliricidia trees in an agroforestry system (spacing 10 × 0.5 m). Decreasing the pruning frequency increases the annual shoot biomass production and enhances the shoot axis:leaf biomass ratio (Marroquín et al. 2005).

When intercropped in cocoa plantations to provide shade, gliricidia trees are often planted at recommended spacing of 9 m × 9 m (Asare and David 2010). On the basis of shoot growth assessment (Table 6), the shoots (shoot axis and leaves grown in 1 year) of the resulting 124 gliricidia trees per hectare would therefore contain 78 kg N ha^{-1}. Using the data of % Ndfa obtained for leaves on the sites 1, 2, 4, and 5 (Fig. 2) and for the shoot axes on site 5 (48%) and assuming that on all sites gliricidia trees had similar growth as on site 1, we can speculate that the annually produced shoots of gliricidia, theoretically suitable to become green manure after pruning, contain from 31 to 38 kg Ndfa ha^{-1} (using the lowest and highest % Ndfa, site 2 wet and site 4, respectively) (Fig. 4). These amounts certainly underestimate the whole tree N fixation of gliricidia as they do not take into account the N stored in roots and in stems.

According to the recent review on mineral nutrition of cocoa (van Vliet and Giller 2017), the N removal by beans and husks average 30 kg N Mg^{-1}. In Ghana, the national on-farm average cocoa bean yields are approximately 0.4 Mg ha^{-1} and as high as 3.36 Mg ha^{-1} if trees are properly managed (Aneani and Ofori-Frimpong 2013). It is therefore reasonable to expect net N

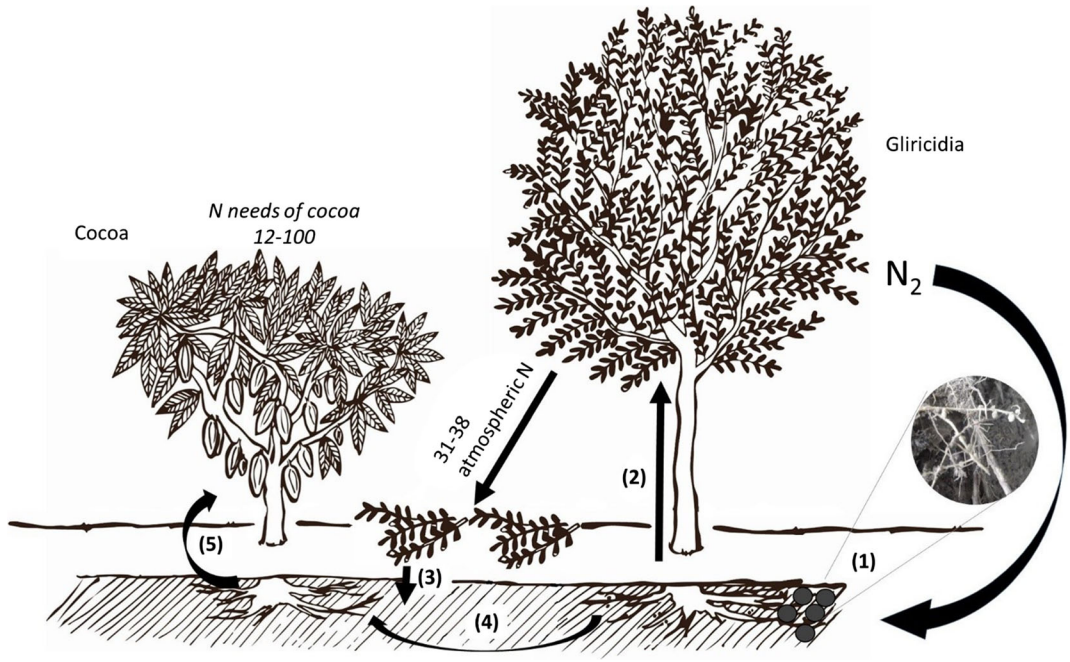

Fig. 4 Schematic of the transfer of the atmospheric N (Ndfa) fixed by *Rhizobium tropici* and *R. etli* associated with gliricidia root nodules to cocoa trees in intercropping systems through pruned shoots left on top of the soil as green manure. (1) Symbiotic N fixation by root nodules; (2) uptake of Ndfa from gliricidia trees and allocation to shoots; (3) Gliricidia shoots pruned and left at the soil surface decompose and release Ndfa that enters the soil; (4) potential direct Ndfa transfer from gliricidia roots to cocoa roots through mycorrhizal network; (5) Ndfa uptake by cocoa roots. Data are in kg N ha^{-1}

removals ranging from 12 to 100 kg N ha^{-1} depending on the yields (Fig. 4). Such values are compatible with the recommended N-fertilizer rates in Ghana and Ivory Coast, often in the range of 58–80 kg N ha^{-1} (van Vliet and Giller 2017).

In our study, the benefit of gliricidia trees in terms of enhanced N availability for cocoa trees grown in the vicinity was supported by the evidence that the $\delta^{15}N$ of cocoa C trees was lower than that of cocoa F trees growing further away from gliricidia trees. The fact that we have not found differences of $\delta^{15}N$ between gliricidia and close cocoa trees is in line with Nygren and Leblanc (2009), who reported that leaves of *Inga edulis* trees had lower $\delta^{15}N$ values than those of cocoa trees growing further away, but similar $\delta^{15}N$ values to close cocoa trees. It should, however, be kept in mind that there are risks associated with an estimate of N transfer from legume to non-legume trees based solely on $\delta^{15}N$ values, due to the unknown extent of the $\delta^{15}N$ isotopic fractionation during the transfer process (Peoples et al. 2015). Similar $\delta^{15}N$ values between a legume and a non-legume tree would in fact apparently suggest that the latter entirely use N from the legume. Gliricidia shoots used as green manure in intercropping systems under tropical climates rapidly decompose at the soil surface and release most of their N content within few months (Handayanto et al. 1994), which is then available for successive uptake by cocoa trees. Further investigation is, however, required to assess to which extent the transfer of the atmospheric N to cocoa trees occurs through decomposition of pruned material or by direct transfer from gliricidia to neighboring cocoa trees via a common mycelial network that, although not yet demonstrated for cocoa and gliricidia, is likely to occur in nature (Kurppa et al. 2010).

In conclusion, we have demonstrated that *Gliricidia sepium* trees are able to take advantage of the association with rhizobial symbionts, to fulfill, at least in part, the N needs of their rapidly growing shoots. In mixed-stand agroforestry systems, with intercropped gliricidia and cocoa trees, the amount of N derived from the atmosphere that could enter the soil if the pruned shoots are used as green manure could diminish the N-fertilizer needs by a minimum of 37% (using 31.4 kg ha^{-1} Ndfa and a fertilizer rate of 80 kg ha^{-1} N) to a maximum of 65% (using 39 kg ha^{-1} Ndfa and a fertilizer rate of 58 kg ha^{-1} N).

Acknowledgements The authors like to thank Prof. Paul Vlek for revising the text and for his suggestions. We are also grateful to the anonymous reviewers of the manuscript for their useful remarks and comments. Thanks also to the Bunso Cocoa College (Ghana) for allowing us to sample from its experimental fields. Many thanks also to the design student Marinetta Gorassini for the drawings of cocoa and gliricidia trees of Fig. 4 and to Dr. Jason Frentress for final editing of the English text. The study has been supported by the UNIBZ project named "MINCO" (Improvement of N nutrition of cocoa intercropped with legume trees). The publication of this work was supported by the Open Access Publishing Fund of the Free University of Bozen-Bolzano.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Acosta-Durán C, Martínez-Romero E (2002) Diversity of rhizobia from nodules of the leguminous tree *Gliricidia sepium*, a natural host of *Rhizobium tropici*. Arch Microbiol 178:161–164. https://doi.org/10.1007/s00203-002-0433-3

Afrifa AA, Ofori-Frimpong K, Appiah MR, et al (2010) Nitrogen, phosphorus and potassium budget under the cocoa ecosystem: produce harvesting phase. SAN JOSÈ COSTA RICA, pp 395–404

Agnolucci M, Cristani C, Battini F, Palla M, Cardelli R, Saviozzi A, Nuti M (2013) Microbially-enhanced composting of olive mill solid waste (wet husk): bacterial and fungal community dynamics at industrial pilot and farm level. Bioresour Technol 134:10–16. https://doi.org/10.1016/j.biortech.2013.02.022

Agnolucci M, Battini F, Cristani C, Giovannetti M (2015) Diverse bacterial communities are recruited on spores of different arbuscular mycorrhizal fungal isolates. Biol Fertil Soils 51:379–389. https://doi.org/10.1007/s00374-014-0989-5

Aneani F, Ofori-Frimpong K (2013) An analysis of yield gap and some factors of cocoa (*Theobroma cacao*) yields in Ghana. Sustain Agric Res 2:117–127. https://doi.org/10.5539/sar.v2n4p117

Aneani F, Anchirinah V, Asamoah M, Owusu-Ansah F (2011) Analysis of economic efficiency in cocoa production in Ghana. Afr J Food Agric Nutr Dev 11:4507–4526. https://doi.org/10.4314/ajfand.v11i1.65877

Anhar A (2005) The role of biological nitrogen fixation in the cacao agroforestry system in Central Sulawesi Indonesia. Cuvillier Verlag, Göttingen, p 108

Anim-Kwapong GJ (2003) Potential of some Neotropical Albizia species as shade trees when replanting cacao in Ghana. Agrofor Syst 58:185–193. https://doi.org/10.1023/A:1026097423351

Apolinário VX d O, Dubeux JCB, Lira M d A et al (2016) Arboreal legume litter nutrient contribution to a tropical silvopasture. Agron J 108:2478–2484. https://doi.org/10.2134/agronj2016.02.0120

Asare R, David S (2010) Planting, replanting and tree diversification in cocoa systems: learning about sustainable cocoa production: a guide for participatory farmer training. Forest & Landscape Denmark, Hørsholm, p 21

Baah F, Anchirinah V, Amon-Armah F (2011) Soil fertility management practices of cocoa farmers in the Eastern Region of Ghana. Agric Biol J N Am 2:173–181. https://doi.org/10.5251/abjna.2011.2.1.173.181

Baffoe-Asare R (2013) Socioeconomic factors influencing adoption of codapec and cocoa high-tech technologies among small holder farmers in central region of Ghana. Am J Exp Agric 3:277–292. https://doi.org/10.9734/AJEA/2013/1969

Bassler R, Hoffmann G, Thun R (1991) Die Untersuchung Von Böden. VDLUFA-Verl, Darmstadt

Bateman AS, Kelly SD (2007) Fertilizer nitrogen isotope signatures. Isot Environ Health Stud 43:237–247. https://doi.org/10.1080/10256010701550732

Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. Nutr Cycl Agroecosyst 57:235–270. https://doi.org/10.1023/A:1009890514844

Chalk PM, He J-Z, Peoples MB, Chen D (2017) $^{15}N_2$ as a tracer of biological N_2 fixation: a 75-year retrospective. Soil Biol Biochem 106:36–50. https://doi.org/10.1016/j.soilbio.2016.12.010

Dirzo R, Young HS, Mooney HA, Ceballos G (2012) Seasonally dry tropical forests: ecology and conservation. Island Press: Made available through hoopla, United States

Elevitch CR, Francis JK (2006) *Gliricidia sepium* (gliricidia). In: Elevitch CR (ed) Species profiles for Pacific Island agroforestry. Permanent Agriculture Resources (PAR), Holualoa Available via http://www.traditionaltree.org. Accessed 29 Nov 2018

Fownes JH, Anderson DG (1991) Changes in nodule and root biomass of *Sesbania sesban* and *Leucaena leucocephala* following coppicing. Plant Soil 138:9–16. https://doi.org/10.1007/BF00011802

Gehring C, Vlek PLG (2004) Limitations of the ^{15}N natural abundance method for estimating biological nitrogen fixation in Amazonian forest legumes. Basic Appl Ecol 5:567–580. https://doi.org/10.1016/j.baae.2004.09.005

Hairiah K, Van Noordwijk M, Cadisch G (2000) Quantification of biological N_2 fixation of hedgerow trees in Northern Lampung. NJAS - Wagening J Life Sci 48:47–59. https://doi.org/10.1016/S1573-5214(00)80004-4

Handayanto E, Cadisch G, Giller KE (1994) Nitrogen release from prunings of legume hedgerow trees in relation to quality of the prunings and incubation method. Plant Soil 160:248–337

Hartemink AE (2005) Nutrient stocks, nutrient cycling, and soil changes in cocoa ecosystems: a review. Adv Agron 86:227–253. https://doi.org/10.1016/S0065-2113(05)86005-5

Hernandez-Lucas I, Segovia L, Martínez-Romero E, Pueppke SG (1995) Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Appl Environ Microbiol 61:2775–2779

Hobbie EA, Högberg P (2012) Nitrogen isotopes link mycorrhizal fungi and plants to nitrogen dynamics. New Phytol 196:367–382. https://doi.org/10.1111/j.1469-8137.2012.04300.x

IFDC (2012) Ghana fertilizer assessment. pp 40 Available via https://ifdcorg.files.wordpress.com/2015/04/ghana-fertilizer-assessment.pdf. Accessed 29 Nov 2018

Isaac ME, Harmand J-M, Lesueur D, Lelon J (2011) Tree age and soil phosphorus conditions influence N2-fixation rates and soil N dynamics in natural populations of *Acacia senegal*. For Ecol Manag 261:582–588. https://doi.org/10.1016/j.foreco.2010.11.011

Khamzina A, Lamers JPA, Vlek PLG (2009) Nitrogen fixation by *Elaeagnus angustifolia* in the reclamation of degraded croplands of Central Asia. Tree Physiol 29:799–808. https://doi.org/10.1093/treephys/tpp017

Kinkema M, Scott PT, Gresshoff PM (2006) Legume nodulation: successful symbiosis through short- and long-distance signalling. Funct Plant Biol 33:707–721. https://doi.org/10.1071/FP06056

Kolavalli S, Vigneri M (2011) Cocoa in Ghana: shaping the success of an economy. In: Yes, Africa can: success stories from a dynamic continent. World Bank, Washington, D.C., pp 201–217

Kurppa M, Leblanc HA, Nygren P (2010) Detection of nitrogen transfer from N2-fixing shade trees to cacao saplings in ^{15}N labelled soil: ecological and experimental considerations. Agrofor Syst 80:223–239. https://doi.org/10.1007/s10457-010-9327-6

Ladha JK, Garrity DP, Peoples MB, Capuno VT, Dart PJ (1993) Estimating dinitrogen fixation of hedgerow vegetation using the nitrogen-15 natural abundance method. Soil Sci Soc Am J 57:732–737. https://doi.org/10.2136/sssaj1993.03615995005700030018x

Leblanc HA, McGraw RL, Nygren P (2007) Dinitrogen-fixation by three neotropical agroforestry tree species under semi-controlled field conditions. Plant Soil 291:199–209. https://doi.org/10.1007/s11104-006-9186-0

Marroquín I, Hernandez J, Martinez A, Barra J (2005) Aboveground biomass production and nitrogen content in *Gliricidia sepium* (Jacq.) Walp. under several pruning regimes. INTERCIENCIA 30:151–158

Martínez-Romero E, Segovia L, Mercante FM et al (1991) Rhizobium tropici, a novel species nodulating *Phaseolus vulgaris* L. beans and </i>Leucaena sp. trees. Int J Syst Bacteriol 41:417–426. https://doi.org/10.1099/00207713-41-3-417

Nick G, Jussila M, Hoste B, Niemi RM, Kaijalainen S, de Lajudie P, Gillis M, de Bruijn FJ, Lindström K (1999) Rhizobia isolated from root nodules of tropical leguminous trees characterized using DNA-DNA dot-blot hybridisation and rep-PCR genomic fingerprinting. Syst Appl Microbiol 22:287–299. https://doi.org/10.1016/S0723-2020(99)80076-9

Nunoo I, Frimpong BN, Frimpong FK (2014) Fertilizer use among cocoa farmers in Ghana: the case of Sefwi Wiawso District. Int J Environ 3:22–31. https://doi.org/10.3126/ije.v3i1.9939

Nxumalo KM, Boshoff FS, Beukes CW, Steenkamp ET, Venter SN (2016) Novel root-nodule bacteria associated with *Vachellia karroo*. S Afr J Bot 103:341–341. https://doi.org/10.1016/j.sajb.2016.02.141

Nygren P, Cruz P (1998) Biomass allocation and nodulation of *Gliricidia sepium* under two cut-and-carry forage production regimes. Agrofor Syst 41:277–292. https://doi.org/10.1023/A:1006064707252

Nygren P, Leblanc HA (2009) Natural abundance of ^{15}N in two cacao plantations with legume and non-legume shade trees. Agrofor Syst 76:303–315. https://doi.org/10.1007/s10457-008-9160-3

Nygren P, Ramírez C (1995) Production and turnover of N_2 fixing nodules in relation to foliage development in periodically pruned *Erythrina poeppigiana* (Leguminosae) trees. For Ecol Manag 73:59–73. https://doi.org/10.1016/0378-1127(94)03505-Q

Nygren P, Cruz P, Domenach AM, Vaillant V, Sierra J (2000) Influence of forage harvesting regimes on dynamics of biological dinitrogen fixation of a tropical woody legume. Tree Physiol 20:41–48

Nygren P, Fernández MP, Harmand J-M, Leblanc HA (2012) Symbiotic dinitrogen fixation by trees: an underestimated resource in agroforestry systems? Nutr Cycl Agroecosyst 94:123–160. https://doi.org/10.1007/s10705-012-9542-9

Nygren P, Leblanc HA, Lu M, Gómez Luciano CA (2013) Distribution of coarse and fine roots of *Theobroma cacao* and shade tree *Inga edulis* in a cocoa plantation. Ann For Sci 70:229–239. https://doi.org/10.1007/s13595-012-0250-z

ÖNORM L 1087 (2012) Chemische Bodenuntersuchungen - Bestimmung von “pflanzenverfügbarem” Phosphor und Kalium nach der Calcium-Acetat-Lactat (CAL)-Methode

Opoku-Ameyaw K, Oppong F, Acheampong K, Amoah F (2012) Long term assessment of the agronomic and economic benefits of cocoa food crop intercropping in the absence of fertilizer application. Am J Exp Agric 2:186–197. https://doi.org/10.9734/AJEA/2012/1015

Partey ST, Quashie-Sam SJ, Thevathasan NV, Gordon AM (2011) Decomposition and nutrient release patterns of the leaf biomass of the wild sunflower (*Tithonia diversifolia*): a comparative study with four leguminous agroforestry species. Agrofor Syst 81:123–134. https://doi.org/10.1007/s10457-010-9360-5

Peoples MB, Chalk PM, Unkovich MJ, Boddey RM (2015) Can differences in 15 N natural abundance be used to quantify the transfer of nitrogen from legumes to neighbouring non-

legume plant species? Soil Biol Biochem 87:97–109. https://doi.org/10.1016/j.soilbio.2015.04.010

Rosendahl L, Vance CP, Miller SS, Jacobsen E (1989) Nodule physiology of a supernodulating pea mutant. Physiol Plant 77:606–612. https://doi.org/10.1111/j.1399-3054.1989.tb05398.x

Rowe EC, van Noordwijk M, Suprayogo D, Hairiah K, Giller KE, Cadisch G (2001) Root distributions partially explain ^{15}N uptake patterns in Gliricidia and Peltophorum hedgerow intercropping systems. Plant Soil 235:167–179. https://doi.org/10.1023/A:1011961409353

Schroth G, Zech W (1995) Above- and below-ground biomass dynamics in a sole cropping and an alley cropping system with *Gliricidia sepium* in the semi-deciduous rainforest zone of West Africa. Agrofor Syst 31:181–198. https://doi.org/10.1007/BF00711725

Shearer G, Kohl D (1986) N_2-fixation in field settings: estimations based on natural ^{15}N abundance. Aust J Plant Physiol 13:699–756. https://doi.org/10.1071/PP9860699

Subramanian P, Dhanapal R, Sanil P et al (2005) Glyricidia (*Glyricidia sepium*) as green manure in improving soil fertility and productivity of coconut under coastal littoral sandy soil. J Plant Crops 33:179–183

Tsai SM, Nodari RO, Moon DH, Camargo LEA, Vencovsky R, Gepts P (1998) QTL mapping for nodule number and common bacterial blight in *Phaseolus vulgaris* L. Plant Soil 204:135–145. https://doi.org/10.1023/A:1004347515127

Tscharntke T, Clough Y, Bhagwat SA, Buchori D, Faust H, Hertel D, Hölscher D, Juhrbandt J, Kessler M, Perfecto I, Scherber C, Schroth G, Veldkamp E, Wanger TC (2011) Multifunctional shade-tree management in tropical agroforestry landscapes - a review: multifunctional shade-tree management. J Appl Ecol 48:619–629. https://doi.org/10.1111/j.1365-2664.2010.01939.x

Unkovich M, Herridge D, Peoples MB et al (2008) Measuring plant-associated nitrogen fixation in agricultural systems. Australian Centre for International Agricultural Research, Canberra, p 258

van Vliet JA, Giller KE (2017) Mineral nutrition of cocoa. Adv Agron 141:185–270. https://doi.org/10.1016/bs.agron.2016.10.017

Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-Denaturing Gradient Gel Electrophoresis. Appl Environ Microbiol 70:4800–4806.