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Natural abundance of ^{15}N in two cacao plantations with legume and non-legume shade trees

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Abstract Natural abundance of ^{15}N was sampled in young and mature leaves, branches, stem, and coarse roots of trees in a cacao (*Theobroma cacao*) plantation shaded by legume tree *Inga edulis* and scattered non-legumes, in a cacao plantation with mixed-species shade (legume *Gliricidia sepium* and several non-legumes), and in a tree hedgerow bordering the plantations in Guácimo, in the humid Caribbean lowlands of Costa Rica. The deviation of the sample ^{15}N proportion from that of atmosphere ($\delta^{15}N$) was similar in non-legumes *Cordia alliodora*, *Posoqueria latifolia*, *Rollinia pittieri*, and *T. cacao*. Deep-rooted *Hieronyma alchorneoides* had lower $\delta^{15}N$ than other non-N_2-fixers, which probably reflected uptake from a partially different soil N pool. *Gliricidia sepium* had low $\delta^{15}N$. *Inga edulis* had high $\delta^{15}N$ in leaves and branches but low in stem and coarse roots. The percentage of N fixed from atmosphere out of total tree N ($\%N_f$) in *G. sepium* varied 56–74%; N_2 fixation was more active in July (the rainiest season) than in March (the relatively dry season). The variation of $\delta^{15}N$ between organs in *I. edulis* was probably associated to ^{15}N fractionation in leaves. Stem and coarse root $\delta^{15}N$ was assumed to reflect the actual ratio of N_2 fixation to soil N uptake; stem-based estimates of $\%N_f$ in *I. edulis* were 48–63%. *Theobroma cacao* below *I. edulis* had lower $\delta^{15}N$ than *T. cacao* below mixed-species shade, which may indicate direct N transfer from *I. edulis* to *T. cacao* but results so far were inconclusive. Further research should address the ^{15}N fractionation in the studied species for improving the accuracy of the N transfer estimates. The $\delta^{15}N$ appeared to vary according to ecophysiological characteristics of the trees.

Keywords *Gliricidia sepium* · *Inga edulis* · ^{15}N fractionation · N_2 fixation · N transfer · Reference species

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Introduction

Versatile legume (Fabaceae) trees may be used for various purposes in tropical agroforestry systems (AFS) like shade trees for perennial crops, production of green manure and fuel wood in alley cropping, soil recovery in enriched fallow systems, fodder, or support trees for climbing crops (Nair 1993). The use of legume trees is motivated by the assumed benefit of symbiotic fixation of atmospheric dinitrogen by the trees for the whole AFS. Both traditional knowledge and research results indicate that the presence of legume trees has beneficial effects on soil

fertility in many tropical AFS (Kass et al. 1997). These benefits include increased soil and microbial C and N content in comparison to annual cropping (Mazzarino et al. 1993; Sierra et al. 2002), increased residual soil N content after legume-enriched fallows in comparison to continuous cropping (Ståhl et al. 2002), and long-term accumulation of C and N in soil in simultaneous AFS (Dulormne et al. 2003; Haggar et al. 1993; Sierra and Nygren 2005). Some legume benefits like increased N mineralization and nitrification rate (Babbar and Zak 1994) may be caused by interaction of increased N availability from symbiotic N_2 fixation and beneficial modification of microclimate below tree canopy.

The actual contribution of symbiotic N_2 fixation to the observed benefits remains debatable. In a review, Mafongoya et al. (2004) cite a range of 14–92% of legume tree N originating from symbiotic N_2 fixation, with large majority of values being between 40% and 80%. Nodulation and symbiotic N_2 fixation rate depend heavily on environmental conditions (Salas et al. 2001) and tree management (Nygren and Ramírez 1995; Nygren et al. 2000). Further, estimating the N contribution by legume trees to N economy of an AFS under field conditions is a methodologically challenging task. It is nowadays generally accepted that the acetylene reduction assay (ARA) may only be used for showing if the plants fix N or not but not for quantification of the absolute N_2 fixation rate (Hunt and Layzell 1993). Constructing a whole-system N balance may be a reliable method (Dulormne et al. 2003) but it requires several measurements on all system components over time, thus requiring a lot of human and financial resources, and long-term experiments.

Methods based on the relationships between the heavy, stable ^{15}N isotope and the common ^{14}N isotope are considered the best yet not perfect way to estimate the percentage of N fixed from atmosphere out of total plant N—an integrating estimate of N_2 fixation capacity by legumes. The main problem of the ^{15}N enrichment methods under field conditions is achieving uniform isotopic labelling of the soil both spatially and temporarily, and selecting a suitable non-N_2-fixing reference species (Chalk and Ladha 1999). This problem is especially important in the case of trees that may scavenge nutrients from a horizontally extensive area and from several soil layers.

The ^{15}N natural abundance method (Shearer and Kohl 1986) overcomes the problems of applying the ^{15}N label but it is even more sensitive to selecting a proper reference species (Domenach 1995). Further, the ^{15}N natural abundance in plants may vary for a number of factors other than N_2 fixation. Soil biological activity alters the isotopic signature of soil (Högberg 1997); plant metabolism tends to discriminate against the heavy ^{15}N isotope (Handley and Raven 1992) resulting in differences between plant organs and species; and different types of mycorrhizae have different effects on plant isotopic signature (Handley et al. 1999; Högberg 1997). Plant metabolism and mycorrhizae affect especially the isotopic signature of non-N_2-fixing plants but may also alter the isotopic relationships in N_2-fixing plants (Roggy et al. 1999; Wheeler et al. 2000). It is recommended that the reference species should grow close to the N_2-fixing plants (Domenach 1995; Roggy et al. 1999). Direct transfer of N fixed from atmosphere from the legume trees to associated crops has recently been observed in some AFS (Sierra and Nygren 2006; Snoeck et al. 2000). Thus, the isotopic signature of the non-N_2-fixing reference near the N_2-fixer may have been directly affected by the N transfer from the latter.

Even considering the constraints listed above, the ^{15}N natural abundance method may be the most practical way to obtain field estimates on N_2 fixation in AFS, if conducted carefully, because it does not require expensive ^{15}N labelled fertilizers and is not sensitive to short-term dilution of the ^{15}N label in the soil (Boddey et al. 2000). In this contribution, we report results of ^{15}N natural abundance sampling in two cacao (*Theobroma cacao*) plantations with legume tree (*Inga edulis*) and mixed-species shade canopy under humid tropical conditions. Reference sampling was also conducted in a tree hedgerow at one side of the cacao plantations. The objectives of the study were to (i) measure the ^{15}N natural abundance in two legume and several non-N_2-fixing tree species in the area during the driest and rainiest season of a year; (ii) identify suitable non-N_2-fixing reference tree species for estimating the percentage of N fixed from atmosphere out of total N ($\%N_f$) in the N_2-fixers; (iii) estimate the $\%N_f$ in the assumed N_2-fixers; and (iv) evaluate possible evidence of direct N transfer from the legume trees to non-N_2-fixing species.

Materials and methods

Field sampling

The study was conducted in two cacao plantations located near to each other in the EARTH University academic farm located in the Caribbean coastal plain of Costa Rica (10°10′ N, 83°37′ W, 64 m a.s.l.), and a tree hedgerow that separates the cacao plantations from an adjacent pasture (Fig. 1). The climatic zone is premontane wet forest basal belt transition (Bolaños and Watson 1993). Average annual rainfall in 1996–2005 was 3,713 mm (Fig. 2). The study year 2006 was somewhat drier than the average with a rainfall of 2,911 mm. The rainfall is quite evenly distributed throughout a year with relatively less rain in March and September. Monthly variation in the rainiest months is higher than in the driest months. The high rainfall and variation in May are strongly affected by three very rainy years (1997, 2002 and 2004) when the May rainfall was ca. 1,000 mm. The annual mean temperature is 25.1°C with very little monthly variation. The soil of the study area is an andic humitropept (Sancho et al. 1989).

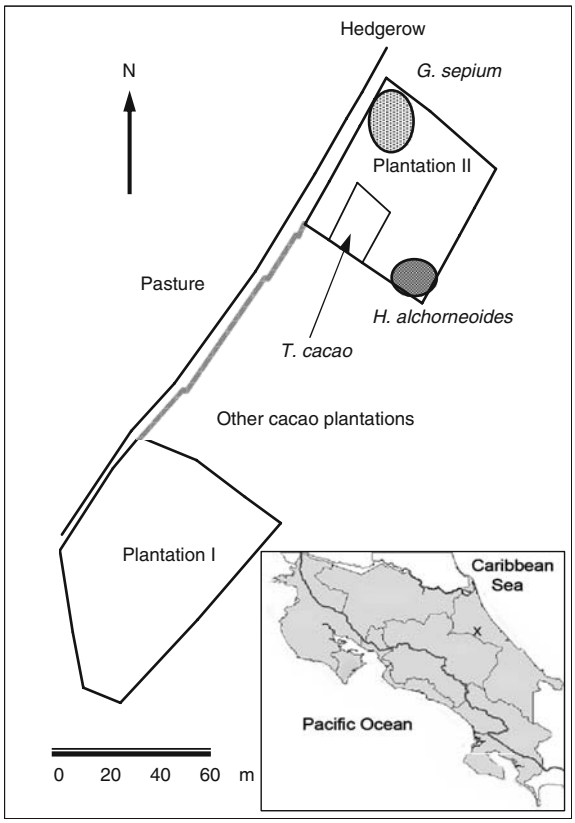

Fig. 1 Map of the study site indicating the relative positions of cacao Plantations I (with *Inga edulis* as shade tree) and II (with several shade tree species), and the adjacent tree hedgerow separating the cacao plantations from a pasture. Approximate positions of *Theobroma cacao*, *Gliricidia sepium*, and *Hieronyma alchorneoides* samplings in Plantation II are shown. Samplings in Plantation I and Hedgerow were distributed throughout the whole area. See Table 1 for a complete listing of tree species sampled. The inset shows the geographic position of the study area

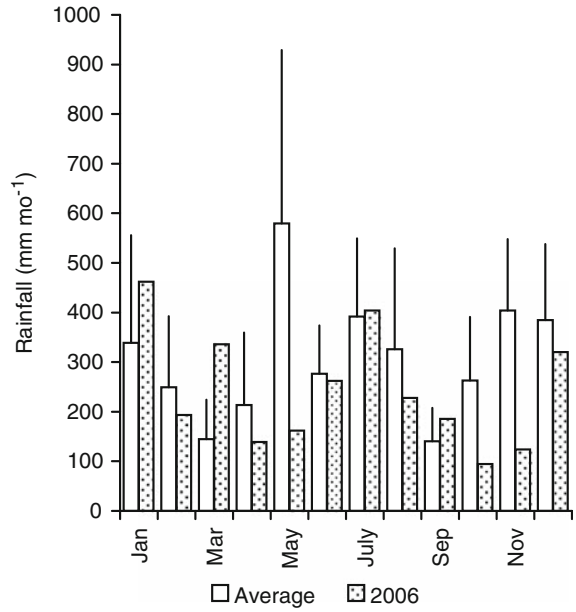

Fig. 2 Average monthly rainfall in the humid tropical study site in Guácimo, Costa Rica, from 1996 to 2005 and during the sampling year 2006. Error bars for the monthly means indicate standard deviation

One of the cacao plantations (hereafter Plantation I; Fig. 1) was established in 1992 using various *T. cacao* clones. It has been cultivated organically since 1997. The plantation size is about 60 × 60 m with *T. cacao* planted in 3 × 3-m spacing. A few scattered *Cordia alliodora* trees were left on the site while establishing the plantation. *Inga edulis* was planted as the main shade tree in 9 × 9-m spacing. Each *I. edulis* replaced a *T. cacao*. By October 2005, the *I. edulis* canopy had become closed and too dense for good cacao production, and the shade trees were thinned to approximate 18 × 18-m spacing, leaving all *C. alliodora*s untouched. *Theobroma cacao* trees were pruned in December 2005, after the major annual harvest of cacao pods. All organic fertilisation

was discontinued in 2005 because the isotopic signature of the fertilizer could not be controlled between preparations of different fertilizer lots.

The other cacao plantation (hereafter Plantation II; Fig. 1) was established in 1995 using matina cultivar of *T. cacao* and industrial fertilizers were applied. The original plantation was partially destroyed by escaped cattle and it was renewed using various *T. cacao* clones in 2002. The clonal trees were planted between the old *T. cacao* rows. The plantation has been managed organically since re-establishment. Old *T. cacao* trees that survived the browsing and various planted banana varieties served as temporal shade, and Peach palm (*Bactris gasipaes* Kunth) was planted as permanent shade. At the time of ^{15}N natural abundance sampling, the palms did not provide any significant shade. Plantation II also contained a mixture of scattered, naturally-established shade trees (Table 1). The ^{15}N natural abundance samples were collected from the old (matina) *T. cacao* trees that were situated at least 15 m from the nearest N_2-fixing shade tree. All organic fertilization was discontinued in 2005.

Two N_2-fixing and five non-N_2-fixing tree species were sampled (Table 1). *Theobroma cacao*s from Plantations I and II were dealt as separate pseudo-species in all analyses. Plant organs sampled were:

- Young leaves: youngest leaf that had reached full size but still had lighter green colour than mature leaves. Depending on species, these leaves were 1–2 weeks old.
- Mature leaves: fully developed, deep-green leaves from lower position of a branch, without marked insect or disease damage.
- Branches: small woody branches that carried leaves.
- Stem: a core sample taken with an increment borer to stem centre at breast height (1.3 m; top of stem in *T. cacao*).
- Coarse roots: a sample from a large structural root at 1–2 m from root collar. Depending on tree size, the sample was taken as a cylinder of whole root or as a core sample passing the whole root diameter.

The sampling was conducted in March and July. These months correspond to the driest and rainiest period of a year in the study site (Fig. 2). *Cordia alliodora* did not have any young leaves in March; otherwise all sample types could be collected from all species in both March and July.

^{15}N natural abundance analyses and calculations

The N isotopic analyses were conducted by isotope ratio mass spectrometry (IRMS) in the Dating Laboratory at the University of Helsinki. The samples were weighted in tin capsules, which were fed by an auto sampler in He–O_2 flow to an element analyzer (Carlo Erba NC2500, Italy) for volatilisation. The volatilised nitrogen oxides were reduced to N_2 by Cu. After reduction, the samples were directed through CO_2 and H_2O traps and a ConFlo III interface (Thermo Scientific, Bremen, Germany) to the IRMS (Delta Advantages, Thermo Scientific). The laboratory

Table 1 Tree species studied and location of sample trees in the humid tropical study site in Guácimo, Costa Rica

Scientific name	Family	Local name	Growth habit	Location
Gliricidia sepium (Jacq.) Kunth ex Walp.	Fabaceae, Papilionoideae	Madero negro	Small, multistem tree	Plantation II (3) and Hedgerow (2)
Inga edulis Mart.	Fabaceae, Mimosoideae	Guaba rabo de mono	Medium-sized tree	Plantation I (5)
Theobroma cacao L.	Sterculiaceae	Cacao	Shrub	Plantations I (5) and II (5)
Cordia alliodora (Ruiz & Pav.) Oken	Boraginaceae	Laurel	Large tree	Plantation I (3)
Hieronyma alchorneoides Allemão	Euphorbiaceae	Pilón	Large tree[a]	Plantation II (3)
Rollinia pittieri Saf.	Annonaceae	Anonillo	Large tree	Hedgerow (3)
Posoqueria latifolia (Rudge) Roem. & Shult.	Rubiaceae	Fruta de mono	Small, multistem tree	Hedgerow (3)

Numbers in parenthesis in the location column indicate the number of sample trees in each location. See Fig. 1 for a map of the locations. Nomenclature is according to the Tropicos data base of the Missouri Botanical Garden (http://www.mobot.org)

[a] The individual trees sampled were young, ca. 10 m high

references used in the analyses were calibrated against international references IAEA-N-1 ($\delta^{15}N = 0.4 \pm 0.2‰$) and IAEA-N-2 ($\delta^{15}N = 20.3 \pm 0.2‰$). Total N content of the samples was determined in the Department of Forest Ecology at the University of Helsinki by dry combustion (Leco CNS-1000, Leco Corp., St. Joseph, MI, USA).

The deviation of the sample ^{15}N proportion from that of atmosphere ($\delta^{15}N$) was calculated (Shearer and Kohl 1986):

$$\delta^{15}N = \frac{{}^{15}N/{}^{14}N_s - {}^{15}N/{}^{14}N_{atm}}{{}^{15}N/{}^{14}N_{atm}} \times 1000‰ \quad (1)$$

where $^{15}N/^{14}N_s$ and $^{15}N/^{14}N_{atm}$ are $^{15}N/^{14}N$ ratios in the sample and atmosphere, respectively. The percentage of N fixed from atmosphere out of total N in the sample ($\%N_f$) was calculated (Shearer and Kohl 1986):

$$\%N_f = \frac{\delta^{15}N_r - \delta^{15}N_f}{\delta^{15}N_r - \delta^{15}N_0} \times 100\% \quad (2)$$

where subscript f refers to the N_2-fixing species growing in the field, r to a non-N_2-fixing reference species growing in the same field as the N_2-fixer, and 0 to the N_2-fixing species growing in a N-free medium. In practice, the $\delta^{15}N_0$ must be determined from potted plants under controlled conditions. We performed the $\%N_f$ calculations using mean $\delta^{15}N$ values for each component of Eq. 2. In that case, the standard error of the $\%N_f$ estimate was calculated (Shearer and Kohl 1986):

$$\%S_f = \left[\frac{\left(\delta^{15}N_f - \delta^{15}N_0\right)^2}{\left(\delta^{15}N_r - \delta^{15}N_0\right)^4} \times S_r^2 + \frac{S_f^2}{\left(\delta^{15}N_r - \delta^{15}N_0\right)^2} + \frac{\left(\delta^{15}N_r - \delta^{15}N_f\right)^2}{\left(\delta^{15}N_r - \delta^{15}N_0\right)^4} \times S_0^2\right]^{\frac{1}{2}} \quad (3)$$

where S_r, S_f, and S_0 refer to the standard error of $\delta^{15}N_r$, $\delta^{15}N_f$, and $\delta^{15}N_0$, respectively.

A preliminary estimate on the possible transfer of N fixed by *I. edulis* to *T. cacao* in Plantation I was calculated by modifying Eq. 2 according to Snoeck et al. (2000): $\delta^{15}N_r$ denoted to *T. cacao* without contact with N_2-fixers (Plantation II) and $\delta^{15}N_f$ denoted to *T. cacao* in contact with N_2-fixers (Plantation I). This modification of Eq. 2 results in the percentage of N of atmospheric origin out of total N ($\%N_a$) in the receiver plant (Sierra and Nygren 2006), not the percentage of all N potentially transferred from *I. edulis* to *T. cacao*.

Inga edulis in N-free medium

Because no information on the $\delta^{15}N_0$ value for *I. edulis* was found in the literature, it was determined from potted seedlings grown in N-free medium. Seeds of *I. edulis* were collected from trees at the EARTH University campus. After removing fresh seed pulp, the seeds were immersed for 12 h in water containing macerated root nodules taken from mature *I. edulis* trees for inoculating the seeds with N_2-fixing bacteria. The inoculated seeds were planted in 5 l plastic pots of autoclaved vermiculite mixed with N-free fertilizer. The seedlings were grown in a greenhouse for 3 month.

At the time of harvest, the seedlings were divided into leaves, woody above-ground parts, coarse roots (diameter $\geq$2 mm), fine roots (<2 mm), and nodules. Only leaves without damage or senescence indications were used for the isotopic analyses. The above-ground woody parts included stem and woody branches because they did not differ in the seedlings. Green soft tissue of branches was excluded from the isotopic analyses because the respective branch parts were not sampled in the field, where they formed a negligible proportion of total branch biomass. The samples were analysed by IRMS in the Dating Laboratory at the University of Helsinki.

Results

^{15}N natural abundance

Rainfall during the 2 weeks preceding the March sampling was 67 mm and during 4 weeks 97 mm, which is relatively little in the humid tropical study site (cf. Fig. 2). Rainfall during 2 and 4 weeks prior to the July sampling was 259 and 530 mm,

respectively. These numbers indicate that the March and July sampling corresponded to the driest and rainiest season of the year, respectively.

The δ^{15}N values varied by species and organ sampled (Fig. 3; Table 2). *Gliricidia sepium* had the lowest δ^{15}N value, typical for a N_2-fixer, in all organs and both seasons. *Inga edulis* had the second lowest δ^{15}N value in stem and coarse roots during both seasons but δ^{15}N values in young and mature leaves were high like in non-N_2-fixing species. The relative differences between organs within a species appeared to be similar in March and July except in *H. alchorneoides* (Fig. 3). *Hieronyma alchorneoides* had lower δ^{15}N values than the other non-N_2-fixers. Student's t-test performed by species and organ for testing the differences in δ^{15}N values between seasons indicated that statistically significant differences occurred in woody tissue only in stem and coarse roots of *H. alchorneoides* and *T. cacao* in Plantation I (both lower in March). Statistically significant differences between seasons were detected in young leaves of *G. sepium* and *H. alchorneoides* (higher in March); young leaves of *I. edulis* and *T. cacao* in Plantation I (lower in March); and in

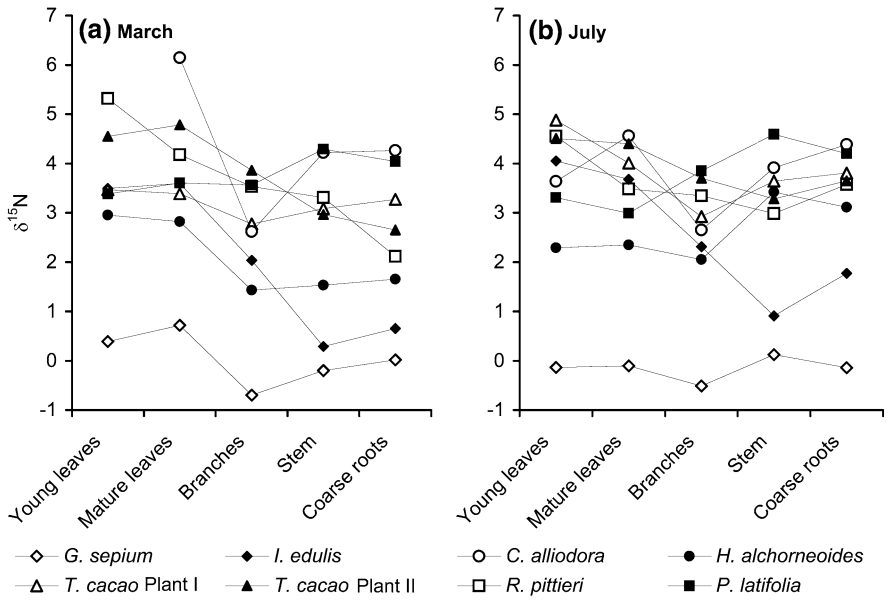

Fig. 3 Mean δ^{15}N values in different organs of trees of two cacao plantations and adjacent hedgerow fence under humid tropical conditions in Guácimo, Costa Rica, during the driest (**a**; March) and rainiest (**b**; August) period of a year

Table 2 Mean of δ^{15}N values in the sampled organs of trees of two cacao plantations and adjacent hedgerow fence under humid tropical conditions in Guácimo, Costa Rica

Tree species	δ^{15}N in				
	Young leaves	Mature leaves	Branch	Stem	Coarse roots
G. sepium	0.13 f, A (0.127)	0.31 e, A (0.181)	−0.60 e, B (0.304)	−0.53 d, A (0.113)	−0.05 e, A (0.157)
I. edulis	3.77 cd, A (0.135)	3.64 c, A (0.186)	2.17 cd, B (0.150)	0.52 d, C (0.230)	0.97 d, C (0.370)
T. cacao Plantation I	4.09 bc, A (0.295)	3.70 c, AB (0.204)	2.84 a, C (0.256)	3.29 b, BC (0.133)	3.54 ab, AB (0.139)
T. cacao Plantation II	4.53 ab, A (0.258)	4.59 b, A (0.183)	3.88 b, B (0.161)	3.10 b, C (0.190)	3.09 bc, C (0.280)
C. alliodora	3.64 cd, B (0.194)	5.51 a, A (0.421)	2.64 bc, C (0.238)	4.06 a, B (0.147)	4.32 a, B (0.308)
H. alchorneoides	2.62 e, A (0.165)	2.58 d, A (0.166)	1.74 d, A (0.183)	2.28 c, A (0.486)	2.38 c, A (0.332)
R. pittieri	4.94 a, A (0.253)	3.83 c, B (0.257)	3.44 a, BC (0.204)	3.18 b, BC (0.176)	2.84 bc, C (0.380)
P. latifolia	3.34 d, C (0.100)	3.30 c, C (0.170)	3.70 a, BC (0.234)	4.44 a, A (0.132)	4.12 a, AB (0.223)

Data is averaged over the samplings in the driest and the rainiest season of a year. Numbers in parentheses indicate the standard error of mean. Means followed by the same lower case letter do not differ significantly within columns and means followed by the same upper case letter do not differ significantly within rows (Duncan's Multiple Range Test at 5%)

mature leaves of *G. sepium* and *C. alliodora* (higher in March).

Because of the relatively few cases of season differences observed, the data for both seasons were pooled for testing the significance of the differences in $\delta^{15}N$ values between species within an organ and between organs within a species using analysis of variance followed by Duncan's Multiple Range Test (Table 2). Statistically significant differences in $\delta^{15}N$ values between young and mature leaves were detected only in *C. alliodora* but it should be noted that this species did not have young leaves in March due to natural phenological variation. In general, all species had higher $\delta^{15}N$ in leaves than in woody tissue. *Gliricidia sepium* had the significantly lowest $\delta^{15}N$ in all organs. *Inga edulis* had the second lowest $\delta^{15}N$ in stem and coarse roots but it did not separate from non-legumes according to the $\delta^{15}N$ in leaves and branches. *Hieronyma alchorneoides* had significantly lower $\delta^{15}N$ than the other non-legume species in all organs but higher than in *G. sepium* (Table 2).

The $\delta^{15}N$ in different organs of *I. edulis* seedlings grown in N-free medium (Table 3) followed the same pattern as in the mature field-grown trees: the leaf sample, which corresponded to a mixture of young and mature leaves from the field, had a relatively high $\delta^{15}N$, followed by coarse roots and stem (cf. Fig. 3). However, all these $\delta^{15}N$ values in N-free medium were negative, typical to a N_2-fixing tree. Fine roots that were not sampled in the field had a $\delta^{15}N$, which did not differ significantly from leaf value, and nodules had the highest $\delta^{15}N$. The nodule sample of the smallest seedling that had $\delta^{15}N$ 1.9‰-units higher than the second highest (6.48 vs. 4.58) was excluded from the mean in Table 3.

Table 3 Mean ± SE of $\delta^{15}N$ values in *Inga edulis* seedlings grown for 3 month in N-free vermiculite

Organ	N	$\delta^{15}N$
Leaves	5	−0.82 ± 0.119 b
Above-ground woody tissues	5	−2.05 ± 0.105 d
Coarse roots	5	−1.58 ± 0.089 c
Fine roots	5	−0.96 ± 0.132 b
Nodules	4	4.30 ± 0.170 a
Weighted mean without nodules	5	−1.09

Means followed by the same letter do not differ significantly (Duncan's Multiple Range Test at 5%). The general mean is weighted by average N content in each organ; nodules are not included

When the $\delta^{15}N$ in mature leaves and stem was plotted against total N concentration in the same organ and season (Fig. 4), three species—*I. edulis*, *G. sepium*, and *C. alliodora*—formed a group with high total N concentration but varying $\delta^{15}N$. *Gliricidia sepium* had the lowest $\delta^{15}N$ in all cases and *C. alliodora* the highest, while $\delta^{15}N$ of *I. edulis* was intermediate in mature leaves and close to that of *G. sepium* in stem. The other five species seemed to be quite similar but *H. alchorneoides* had the significantly lowest $\delta^{15}N$ of this group, except in stem in July (Duncan's MRT at 5%). The $\delta^{15}N$ of *C. alliodora* did not differ significantly from other non-N_2-fixing species than *H. alchorneoides*. Following the criteria proposed by Roggy et al. (1999), *I. edulis* would be classified as a non-N_2-fixer according to mature leaf characteristics but a N_2-fixer according to stem characteristics. All non-legumes appeared to be non-N_2-fixers.

Estimation of N_2 fixation by *I. edulis* and *G. sepium* and transfer of fixed N to *T. cacao*

Estimates of $\%N_f$ were calculated for *I. edulis* and *G. sepium* based on mature leaf and stem $\delta^{15}N$ values. Based on Fig. 4, a non-N_2-fixing reference group was formed using the average $\delta^{15}N$ of *C. alliodora*, *T. cacao* in Plantations I and II, *R. pittieri*, and *P. latifolia* for $\%N_f$ estimations. *Hieronyma alchorneoides* was excluded from $\%N_f$ estimations because its low $\delta^{15}N$ values may indicate that it uses a different soil N pool than the other non-legumes (Roggy et al. 1999). Thus, it may be an unsuitable reference species for estimating $\%N_f$ in the legumes (Domenach 1995; Högberg 1997; Shearer and Kohl 1986). The $\%N_f$ estimates for *I. edulis* were calculated using this group and the non-N_2-fixers of Plantation I, *C. alliodora* and *T. cacao* separately as reference (Table 4). The $\%N_f$ estimates of *I. edulis* based on stem $\delta^{15}N$ in July were almost the same using any of the references, while *C. alliodora* produced higher estimates than *T. cacao* or the non-N_2-fixing group in March. The $\%N_f$ estimates based on mature leaves were low, except in March using *C. alliodora* as the reference species. However, even in this case, the $\%N_f$ estimate based on mature leaves was about half of the estimate based on stem $\delta^{15}N$

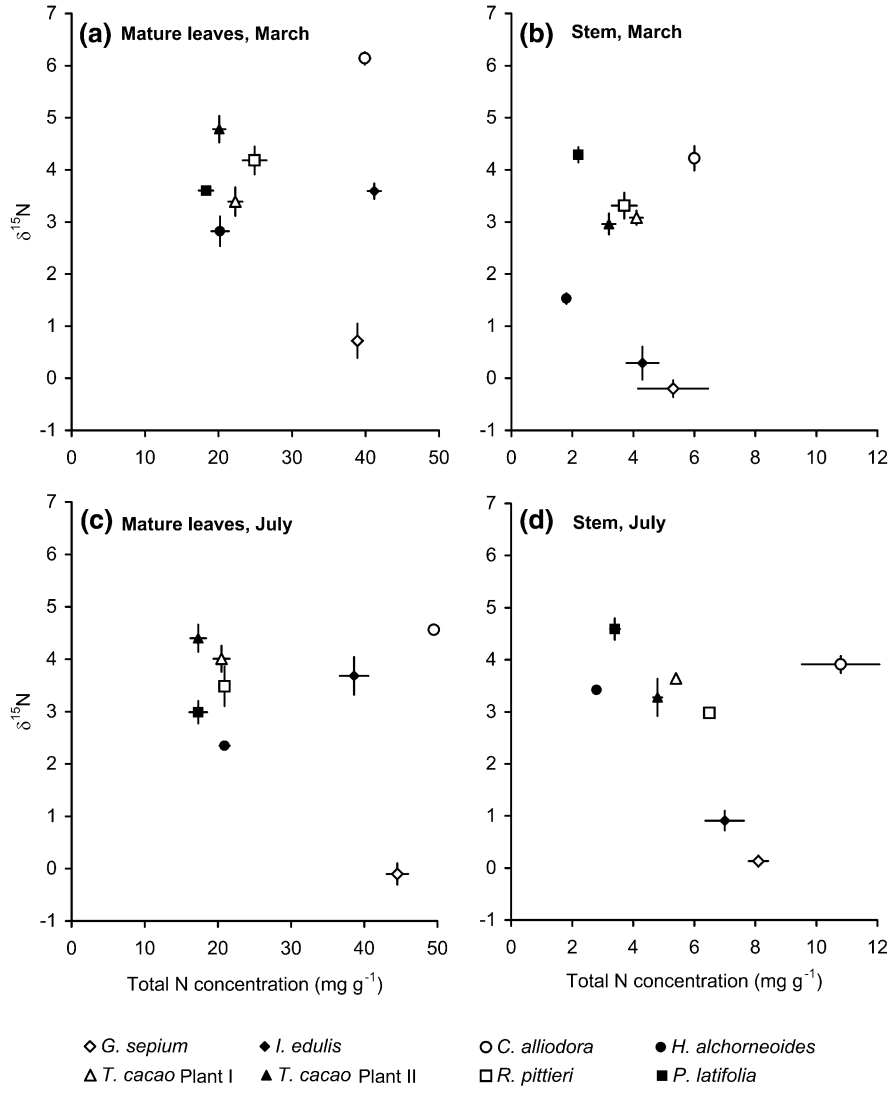

Fig. 4 Mean $\delta^{15}N$ value plotted against mean total N concentration in mature leaves during the driest month, March (**a**), and the rainiest month, July (**c**), and stem in March (**b**) and July (**d**) of trees in two cacao plantations and adjacent hedgerow fence under humid tropical conditions in Guácimo, Costa Rica. The error bars indicate standard error of mean

Table 4 Mean (±SE) percentage of N fixed from atmosphere out of total N in *Inga edulis* growing as shade tree in the cacao Plantation I calculated on the basis of $\delta^{15}N$ values in mature leaves and stem

Reference species	Estimated $\%N_f$ in mature leaves		Estimated $\%N_f$ in stem	
	March	July	March	July
T. cacao Plantation I	−5.0 ± 7.8	6.8 ± 8.9	54.5 ± 6.4	48.0 ± 3.5
C. alliodora Plantation I	36.6 ± 2.5	16.4 ± 6.7	62.7 ± 5.4	50.3 ± 3.6
Mean of non-fixing group[a]	14.6 ± 5.0	5.1 ± 8.4	57.6 ± 6.0	48.5 ± 3.8

[a] *Cordia alliodora*, *Theobroma cacao* in Plantations I and II, *Rollinia pittieri*, and *Posoqueria latifolia*

(Table 4). The stem-based $\%N_f$ estimates suggest active N_2 fixation by *I. edulis* while leaf-based estimates suggest that it would not fix N_2.

Estimates of $\%N_f$ were calculated for *G. sepium* using *T. cacao* growing in the same Plantation (II) and the non-N_2-fixing group as reference. The $\delta^{15}N_0$

Table 5 Mean (±SE) percentage of N fixed from atmosphere out of total N in *Gliricidia sepium* in the cacao Plantation II calculated on the basis of $\delta^{15}N$ values in mature leaves and stem

Reference species	Estimated $\%N_f$ in mature leaves		Estimated $\%N_f$ in stem	
	March	July	March	July
T. cacao Plantation II	59.3 ± 5.6	69.6 ± 4.5	62.8 ± 5.2	59.0 ± 5.1
Mean of non-fixing group[a]	56.5 ± 5.9	67.2 ± 4.7	66.1 ± 4.6	62.0 ± 3.8

[a] *Cordia alliodora*, *Theobroma cacao* in Plantations I and II, *Rollinia pittieri*, and *Posoqueria latifolia*

in leaves of *G. sepium*, −2.07 (Nygren et al. 2000), was also used for stem-based estimates because no information on $\delta^{15}N_0$ for other organs was available and between-organ differences in $\delta^{15}N$ of *G. sepium* appeared to be small (Fig. 3). The results based on both references and organs were quite similar (Table 5). The leaf-based $\%N_f$ estimate was about 10%-units higher in July than in March against both references while season differences were small in stem-based estimates. All $\%N_f$ values clearly indicated N_2 fixation (Table 5).

The effect of organ used for $\%N_f$ estimates was studied by calculating the $\%N_f$ estimate of *G. sepium* against the non-N_2-fixing reference group for all organs separately (Fig. 5). Most organs produced roughly similar $\%N_f$ (ca. 60–65% with extremes of 56% and 74%). The estimates in July were higher for both leaf types and coarse roots and lower for branch- and stem-based calculations (Fig. 5). The $\delta^{15}N$ of the reference group was about the same in March and July in all organs, and the seasonal differences in $\%N_f$ were mainly caused by variation of $\delta^{15}N$ in *G. sepium*.

Because *T. cacao* had significantly lower $\delta^{15}N$ values in Plantation I with *I. edulis* shade trees than in Plantation II without contact with legume trees (Table 2) preliminary estimates on the possible transfer of N fixed by *I. edulis* to *T. cacao* in Plantation I were calculated using $\delta^{15}N$ in mature leaves and stem (Table 6). The leaf-based estimates indicated potential transfer of N fixed by *I. edulis* to *T. cacao* in both March and July. Stem-based estimates suggested that N transfer would not occur.

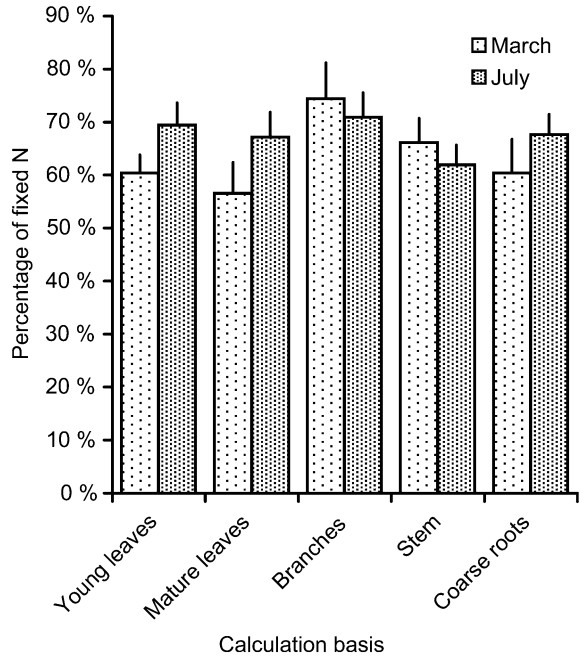

Fig. 5 Mean percentage of N fixed from atmosphere out of total N in different organs of *Gliricidia sepium* growing as a shade tree in a cacao plantation with mixed-species shade stratum (Plantation II) under humid tropical conditions in Guácimo, Costa Rica. The mean $\delta^{15}N$ of four non-fixing tree species (*Cordia alliodora*, *Rollinia pittieri*, *Posoqueria latifolia*, and *Theobroma cacao*) was used as the reference value for each organ. The error bars indicate standard error of mean

Table 6 Mean (±SE) percentage of N of atmospheric origin out of total N in *Theobroma cacao* growing below *Inga edulis* shade trees in the cacao Plantation I in March (the driest season) and July (the rainiest season)

Month	Estimated $\%N_a$ in	
	Mature leaves	Stem
March	24.9 ± 6.1	−2.4 ± 5.0
July	7.5 ± 6.6	−6.8 ± 7.3

Discussion

Natural abundance of ^{15}N, measured as $\delta^{15}N$, did not vary much between the non-N_2-fixing trees

C. alliodora, *P. latifolia*, *R. pittieri*, and *T. cacao* (Fig. 3; Table 2). The $\delta^{15}N$ values were typical to tropical forests (Martinelli et al. 1999). Differences between organs followed the same pattern in this group, i.e. the highest $\delta^{15}N$ in leaves and slightly lower values in woody tissue. The $\delta^{15}N$ in all organs of *G. sepium* was typical for a N_2-fixing tree (Roggy et al. 1999; Yoneyama et al. 1993). The ratio of total N concentration to $\delta^{15}N$ in *P. latifolia*, *R. pittieri*, and *T. cacao* (Fig. 4) was also typical to non-N_2-fixing trees and the relationship in *G. sepium* was typical to a N_2-fixer (Roggy et al. 1999).

Two of the non-legume trees differed from the general pattern of the non-N_2-fixing group: *C. alliodora* had a high leaf total N concentration (Fig. 4) and *H. alchorneoides* had significantly lower $\delta^{15}N$ than the other non-N_2-fixing trees (Table 2). *Cordia alliodora* also had higher yet not significantly different $\delta^{15}N$ than the other non-N_2-fixers. Differences in $\delta^{15}N$ may reflect variations between N sources available for different species and/or distinct mycorrhizal symbionts (Boddey et al. 2000; Handley et al. 1999; Högberg 1997). Nitrate reduction discriminates against ^{15}N (Handley and Raven 1992) causing ^{15}N depletion in plants depending heavily on NO_3^- for N supply. Ectomycorrhizae tend to deplete host plants of ^{15}N (Högberg 1997) while arbuscular mycorrhizae (AM) tend to slightly enrich their hosts by ^{15}N (Handley et al. 1999).

Roots of *I. edulis* and *T. cacao* were colonised by AM in the study site (Iglesias et al. 2007). Soil fungal community in *C. alliodora* and *H. alchorneoides* plantations in Sarapiquí, ca. 50 km NW from the study site under similar climatic conditions, imply AM symbiosis although actual root colonisation was not analysed (Lovelock and Ewel 2005). *Gliricidia sepium* forms AM symbiosis (Okon et al. 1996). No information on mycorrhizal symbioses of any *Rollinia* or *Posoqueria* sp. could be found. Thus, mycorrhizal differences probably do not explain the observed lower $\delta^{15}N$ in *H. alchorneoides*.

Both *I. edulis* and *T. cacao* had superficial rooting pattern in the study site (Nygren et al. 2007) and *G. sepium* seems to root superficially under humid tropical conditions (Rowe et al. 2001; Salas et al. 2004). Root system of *C. alliodora* was observed to be superficial in Sarapiquí in a plantation with abundant understorey vegetation while root system of *H. alchorneoides* was deeper (Haggar and Ewel 1997). Deep rooting pattern of *H. alchorneoides* was also observed in a natural forest and in a pure plantation in Sarapiquí (Arnáez and Moreira 2006). Thus, we assume that *H. alchorneoides* had access to a different soil N pool than the other studied species, which affected its $\delta^{15}N$. This hypothesis could not be fully verified because information was not available on rooting patterns of all species but it was considered strong enough to exclude *H. alchorneoides* from the non-N_2-fixing reference group used for estimating $\%N_f$. *Cordia alliodora* was considered a valid reference species because it appeared to form AM symbiosis and have similar rooting pattern as the legume trees *I. edulis* and *G. sepium*.

The legume tree *I. edulis* had a strange $\delta^{15}N$ profile with high values, typical to non-N_2-fixing trees, in leaves and branches and almost as low $\delta^{15}N$ as *G. sepium* in stem and coarse roots (Fig. 3). High foliar $\delta^{15}N$ of *I. edulis* has also been reported earlier (Roggy et al. 1999; Yoneyama et al. 1993). In French Guiana, *I. edulis* with high foliar $\delta^{15}N$ was nodulated and the nodules showed nitrogenase activity in the acetylene reduction assay (Roggy et al. 1999). *Inga edulis* has also been shown to fix N_2 using ^{15}N enrichment method under greenhouse (Leblanc et al. 2005) and semi-controlled field conditions close to our study site (Leblanc et al. 2007); however, all studied individuals did not fix N_2 in the field. The survival of *I. edulis* in the N-free medium of this study also indicated that it is a N_2-fixer (Table 3).

Thus, *I. edulis* may be considered a confirmed N_2-fixer and its $\delta^{15}N$ profile is probably caused by dissimilar ^{15}N fractionation processes in different organs. The $\delta^{15}N$ between organs of the seedlings grown in N-free medium followed the same pattern as in the Plantation I (Table 3; Fig. 3), i.e. the lowest values in stem and coarse roots. The same profile has also observed in other N_2-fixing trees (reviewed by Boddey et al. 2000) and in both *G. sepium* and non-N_2-fixing trees in this study (Fig. 3). Nodules had the highest $\delta^{15}N$ in the *I. edulis* seedlings grown in N-free medium (Table 3), which seems to be a typical feature of N_2-fixing trees (Boddey et al. 2000). Although the tendency of lower $\delta^{15}N$ in stems than leaves was observed in other N_2-fixing and non-N_2-fixing trees, the difference between leaves and woody tissue in *I. edulis* was the most pronounced. In the ^{15}N enrichment experiment, stem of *I. edulis* had lower ^{15}N atom excess than leaves but the differences

were proportionally equal to differences observed in the non-N_2-fixing reference tree *Vochysia guatemalensis* Donn. Sm. (Leblanc et al. 2007) indicating that strong isotopic enrichment of the growth medium surpasses the effects of fractionating processes. High foliar $\delta^{15}N$ was also observed in some other, but not all, nodulating *Inga* spp. (Koponen et al. 2003; Roggy et al. 1999), which suggests that this characteristic may be associated to some subgroups of the large genus.

Theobroma cacao had significantly lower $\delta^{15}N$ in Plantation I with *I. edulis* shade trees than in Plantation II without contact with legume trees (Table 2; Fig. 3). The estimation of $\%N_a$ using the modified Eq. 2 is based on the assumption that the isotopic signature of the N fixed from atmosphere by *I. edulis* does not change if it is directly transferred to *T. cacao*, e.g. via common mycorrhizal network or root exudates of *I. edulis* absorbed by *T. cacao* (He et al. 2003). The isotopic signature of N mineralised from decomposing leaves and fine roots is strongly altered by microbiological processes of the soil (Boddey et al. 2000; Handley and Raven 1992; Högberg 1997). Thus, N released by *I. edulis* to soil through the complete mineralization process and reabsorbed by *T. cacao* would have the general soil isotopic signature. Leaf-based estimates of $\%N_a$ in *T. cacao* in Plantation I suggested small but significant direct N transfer from *I. edulis* in both seasons (Table 6). However, the results are still inconclusive because of differences between seasons and organs. Direct N transfer via common mycorrhizal networks in the studied plantation may be envisioned because both species were colonised by the same AM morphospecies (Iglesias et al. 2007). Thus, the question requires further research, in which the apparent ^{15}N fractionation processes will be carefully considered.

Statistically significant differences in $\delta^{15}N$ between seasons were observed mainly in young leaves (Fig. 3); higher values were measured in July for *I. edulis* and *T. cacao* in Plantation I. Opposite was observed in *G. sepium* and *H. alchorneoides*. This may reflect variation in plant-available N pools between the driest and rainiest season of a year and higher N_2 fixation rate of *G. sepium* under humid conditions. Leaf $\delta^{15}N$ tends to respond most rapidly to these changes (Domenach 1995). However, significant differences in stems and coarse roots of *T. cacao* in Plantation I and *H. alchorneoides* were somewhat unexpected because the $\delta^{15}N$ in these organs integrates effects of long-term changes in the $\delta^{15}N$ of N sources. The stem of cultivated *T. cacao* is relatively small—most above-ground woody tissue is in morphological branches—and the *H. alchorneoides* individuals sampled were quite young. Thus, it is possible that the small stem of *T. cacao* reacts rapidly to changes in the available N sources. The stems of *H. alchorneoides* were almost entirely of sapwood, and it may be envisioned that large stems are stabilised by the inactive heartwood rather than the physiologically active sapwood.

The estimates of $\%N_f$ for *I. edulis* calculated on the basis of stem $\delta^{15}N$ (Table 4) were about the same as the estimates based on ^{15}N enrichment of growth medium, 57% (Leblanc et al. 2007). Leaf $\delta^{15}N$ did not seem to be a reliable basis for estimating $\%N_f$ in *I. edulis* when the ^{15}N natural abundance method was used. The stem-based estimates were quite similar with various reference trees. Leaf- and stem-based estimates of $\%N_f$ for *G. sepium* were close to each other's independently of reference plant used but seasonal variation appeared to be higher in leaf-based estimates (Table 5). It may be assumed that leaf-based estimates better reflect seasonal variation in N_2 fixation because leaf $\delta^{15}N$ tends to respond more rapidly to changes in N_2 fixation than other organs (Domenach 1995); the $\delta^{15}N$ in stem integrates effects of long-term N_2 fixation. Estimates of $\%N_f$ calculated on the basis of different organs of *G. sepium* were quite close to others (Fig. 5), which indicates that relatively reliable $\%N_f$ estimates can be made for this species by collecting leaves only. The same was observed in ^{15}N enrichment-based estimates for *I. edulis* and two *Erythrina* L. spp. (Leblanc et al. 2007). The $\%N_f$ estimates for *G. sepium* were close to values found in other studies (e.g. Nygren et al. 2000; Peoples et al. 1996).

Concluding remarks

General mean of $\delta^{15}N$ in the non-N_2-fixing trees in the studied cacao plantations, 3.67‰, was very close to the average value of various tropical forests, 3.7‰ (Martinelli et al. 1999). Little variation was observed between the non-N_2-fixing trees *C. alliodora*, *P. latifolia*, *R. pittieri*, and *T. cacao*. Variation between

organs within a species was more pronounced than general variation between these species. *Hieronyma alchorneoides* had significantly lower $\delta^{15}N$ than the other non-N_2-fixers, which probably reflected use of a different soil N pool; it had the deepest rooting pattern of the species, for which information was available. Mycorrhizal differences were assumed to be unimportant in the study site; all species, for which information was available, formed AM symbiosis. The $\%N_f$ of *G. sepium* varied between 56% and 74%, depending on season and organ used for estimation. It may be assumed that coarse roots and stem $\delta^{15}N$ of *I. edulis* reflected the ratio of N_2 fixation to soil N uptake and the high leaf values were altered by fractionation due to plant metabolism. Some transfer of fixed N from *I. edulis* to *T. cacao* may occur but results so far are inconclusive. Further research on N transfer in this AFS should carefully consider the effects of ^{15}N fractionation within trees. The $\delta^{15}N$ appeared to vary according to ecophysiological characteristics of the trees in the cacao plantations.

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