Ecology and Development Series No. 27, 2005

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The role of biological nitrogen fixation in the cacao agroforestry system in Central Sulawesi Indonesia

Cuvillier Verlag Göttingen

To my wife Rama, and our children Ismail, Abdurrahman, and Nurul

ABSTRACT

A comparative evaluation of the ^{15}N enrichment method (^{15}NEM) and the ^{15}N natural abundance method ($^{15}NNAM$) was conducted in two eight-year-old cacao agroforestry systems (Kaduwaa and Makmur) in Central Sulawesi, Indonesia. It was tested whether both methods could be used to estimate the proportion of N derived from atmospheric N_2 (%Ndfa) by *Gliricidia sepium* (Jacq.) Walp. This was done by i) measuring the variability of ^{15}N with soil depth and time, ii) measuring biological nitrogen fixation with different reference plants as well as at different times of the season, and iii) determining litterfall input and aboveground biomass of Gliricidia in the system.

There was no agreement between both methods on the %Ndfa estimates based on the means as well as on individual paired comparision. Only one out of four times of sampling both methods resulted in similar estimates. The %Ndfa estimate with *Theobroma cacao* and *Coffea arabica* as reference plants was 22 to 33 % higher calculated on the basis of the ^{15}NEM than on the $^{15}NNAM$.

After the enrichment with $^{15}NH_4{}^{15}NO_3$, the atom% ^{15}N excess in the soil declined rapidly. There was little lateral movement of ^{15}N fertilizer in the soil. The atom% ^{15}N excess in the leaves of reference plant (0.11-0.32 % in Kaduwaa and 0.04-0.30 % in Makmur) was significantly higher than in the leaves of Gliricidia (0.06-0.10% in Kaduwaa and 0.03-0.07% in Makmur). Based on these data, the %Ndfa of Gliricidia ranges between 53 and 57 % in Kaduwaa and between 29 and 56 % in Makmur. In Kaduwaa, time of sampling affected the %Ndfa estimates, whereas the reference plants did not have a significant influence. On the contrary, in Makmur, reference plant affected the estimates, but not the time of sampling. There remained an uncertainty in the accuracy of the %Ndfa estimate related to the changes of ^{15}N with time and depth coupled with the possibility of different patterns of uptake of ^{15}N between Gliricidia and reference plants.

The natural abundance level of total soil N expressed by the $\delta^{15}N$ value ranged between 6.2-7.9 ‰ and 7.4-8.8 ‰ in Kaduwaa and Makmur, respectively. It showed little variation at a soil depth of 30 to 150 cm whereas the top-soil layer (0–10 cm) was less enriched suggesting a dilution by atmospheric N_2. The $\delta^{15}N$ value of fixing and reference plants depended upon the plant species, the time of sampling, the plant parts as well as the site. In Kaduwaa the $\delta^{15}N$ value of leaves of Gliricidia ranged between 2.4 and 5.3 ‰ and that of leaves of the reference plants between 3.3 and 7.7 ‰. In Makmur this was 1.2-3.7 ‰ for Gliricida and 2.3-9.2 ‰ for the reference plants. Based on these data, in Kaduwaa Gliricidia derived 31 to 34 % of its N by biological nitrogen fixation. In Makmur this was 32-55 %. In Kaduwaa, time of sampling but not the reference plant species affected the %Ndfa estimate. In Makmur, both, reference plant and time of sampling, affect the %Ndfa estimate.

The biological nitrogen fixation in the system contributed around 13-22 kg N ha^{-1} yr^{-1} as standing biomass of Gliricidia and 28-47 kg N ha^{-1} yr^{-1} as recycled residues into the soil. Consequently, Gliricidia plays a major role in maintaining the N balance in the cacao agroforestry system. The N balance in the system ranged from -15 to +17 kg ha^{-1} yr^{-1} depending on the respective quantities used for calculation. If the system was to be converted to a cacao monoculture, which is a practice performed by some farmers in the region, to maintain the soil fertility at the current level, farmers would have to invest 36-38 € for nitrogen-fertilizer ha^{-1} yr^{-1}. Therefore, improving management practices in traditional cacao agroforestry system is a better option than converting to cacao 'monoculture' plantation system.

Die Rolle der biologischen Stickstofffixierung in dem Kakao-Agroforstsystem in Zentralsulawesi Indonesien

KURZFASSUNG

Über den Zeitraum von zwei Jahren wurde in zwei achtjährigen Kakaoagroforstsystemen (in Kaduwaa and Makmur) in Zentralsulawesi, Indonesien, eine vergleichende Untersuchung zur ^{15}N-Anreicherungsmethode (^{15}NEM) und ^{15}N *natural abundance*-Methode (^{15}NNAM) durchgeführt. Es wurde geprüft, ob beide Methoden zur Berechnung des Anteils des aus der Atmosphäre entnommenen N_2 (%Ndfa) durch *Gliricidia sepium* (Jacq.) Walp eingesetzt werden können. Hierbei wurde i) die Variabilität des Vorkommens von ^{15}N im Boden über die Tiefe und die Zeit untersucht, ii) die biologische Stickstofffixierung mit verschiedenen nicht-fixierenden Pflanzen zu verschiedenen Jahreszeiten gemessen und iii) die Menge der Streu und der oberirdischen Biomasse von Gliricidia im System bestimmt.

Die vergleichenden Untersuchungen ergaben keine Übereinstimmung zwischen beiden Methoden hinsichtlich der %Ndfa-Werte; weder auf Grundlage der Mittelwerte noch über einen Vergleich der Einzelwerte. Nur bei einem von vier Beprobungszeitpunkten ergaben die beiden Methoden ähnliche Ergebnisse. Der %Ndfa-Wert mit *Theobroma cacao* und *Coffea arabica* als nicht-fixierende Referenzpflanzen erzielte mit der ^{15}NEM um 22 bis 33 % höhere Werte als mit der ^{15}NNAM.

Nach Anreicherung mit $^{15}NH_4{}^{15}NO_3$ im Zuge der Anwendung der ^{15}NNAM nahm der Atom% ^{15}N-Überschuss im Boden nach kurzer Zeit rapide ab. Eine laterale Verteilung des ^{15}N-Düngers im Boden war kaum zu beobachten. Der Atom% ^{15}N-Überschuss in den Blättern der nicht-fixierenden Referenzpflanzen (0.11-0.32 % in Kaduwaa und 0.04-0.30 % in Makmur) war nicht signifikant höher als der in den Blättern von Gliricidia (0.06-0.10% in Kaduwaa und 0.03-0.07% in Makmur). Auf der Grundlage dieser Ergebnisse lag der %Ndfa von Gliricidia in Kaduwaa zwischen 53 und 57 % und in Makmur zwischen 29 und 56 %. In Kaduwaa beeinflusst der Zeitpunkt der Probenahme die %Ndfa-Werte, während keine signifikante Wirkung durch die Referenzpflanzen beobachtet wird. Im Gegenteil dazu beeinflusste in Makmur nur die Art der Referenzpflanze die Werte, nicht aber der Zeitpunkt der Probenahme. Da der mineralische ^{15}N Gehalt im Boden sich über die Zeit und mit der Bodentiefe änderte, bleibt die Bestimmung der %Ndfa im Hinblick auf die Möglichkeit einer unterschiedlichen ^{15}N-Aufnahme von Gliricidia und den nicht-fixierenden Referenzpflanzen mit Unsicherheiten behaftet.

Das *natural abundance*-Niveau von Gesamt-N im Boden angegeben durch den δ^{15}N-Wert lag in Kaduwaa zwischen 6.2-7.9 ‰ und in Makmur zwischen 7.4-8.8 ‰. Der Wert veränderte sich in Bodentiefen von 30 bis 150 cm kaum. Die oberen 0-10 cm enthielten weniger N, was auf eine mögliche Verdünnung durch atmosphärischen N hinweist. Der δ^{15}N-Wert der fixierenden bzw. nicht-fixierenden Referenzpflanzen hing von Pflanzenart, Probenahmezeitpunkt und Standort ab. In Kaduwaa lag der δ^{15}N-Wert der Blätter von Gliricidia zwischen 2.4 und 5.3 ‰ und der Wert der nicht-fixierenden Pflanzen zwischen 3.3 und 7.7 ‰. In Makmur betrugen diese Werte für Gliricidia 1.2-3.7 ‰ und für die nicht-fixierenden Referenzpflanzen 2.3-9.2 ‰. Folglich erhielt Gliricidia in Kaduwaa 31 bis 34 % und in Makmur 32-55 % seines aufgenommenen Stickstoff durch biologische N-Fixierung. In Kaduwaa beeinflusst der

Probenahmezeitpunkt, jedoch nicht die Referenzpflanze den %Ndfa-Wert. In Makmur beeinflusste sowohl Probenahmezeitpunkt als auch Referenzpflanze den %Ndfa-Wert.

Die biologische Stickstofffixierung im System betrug 13-22 kg N ha^{-1} $Jahr^{-1}$ als oberirdische Biomasse im Bestand der Gliricidiabäume und 28-47 kg N ha^{-1} $Jahr^{-1}$ als Laubstreu. Folglich spielte Gliricidia in den Kakaoagroforstsystemen eine wichtige Rolle bei der Aufrechterhaltung eines ausgeglichenen N-Haushalts. Der N-Haushalt im System betrug -15 bis +17 kg ha^{-1} $Jahr^{-1}$ abhängig von der zur Berechnung eingesetzten Fixierungsleistung. Immer häufiger wird von Bauern in der Region Gliricidia gerodet und das das System in eine Kakaomonokultur umgewandelt. Um das aktuelle Nährstoffniveau des Bodens für solch ein System aufrechtzuerhalten, müssten die Bauern 36-38 € ha^{-1} yr^{-1} in Stickstoffdünger investieren. Daher ist eine optimierte Bewirtschaftungspraxis in traditionellen Kakaoagroforstsystemen eine bessere Option als die Umwandlung in Monokultur-Plantagesysteme.

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Abbreviations and Symbols

%Ndfa	Proportion of N derived from atmospheric N_2
µg	Microgram
µl	Microliter
^{15}NEM	^{15}N enrichment method
^{15}NNAM	^{15}N natural abundance method
ANOVA	Analysis of variance
BD	Bulk density of the soil
BNF	Biological nitrogen fixation
BS	Base saturation
C	Carbon
CEC	Cation exchange capacity
D	Diameter
DM	Dry matter
ECEC	Effective cation exchange capacity
g	Gram
ha	Hectar
kg	Kilogram
m asl	Meter above sea level
M	Mollar
Mg	Megagram (ton)
mg	Milligram
ml	Milliliter
mm	Millimeter
mo	Month
N	Nitrogen
NFTs	Nitrogen fixing trees
NH_4^+	Ammonium
NO_3^-	Nitrate
^oC	Degree Celsius
SOM	Soil organic matter
STORMA	Stability of Tropical Rain Forest Margin
WAP	Week after planting
yr	Year
$\delta^{15}N$	One per thousand deviations from the natural ^{15}N abundance of atmospheric N_2

1 INTRODUCTION

Current forest cover estimates for Indonesia range from 0.9 to 1.2 million km^2, or 48 to 69% of Indonesia's total land area of 1.9 million km^2. This represents 10% of all tropical forests in the world and nearly half of tropical Asia's remaining forest (FAO 2001a). However, the annual deforestation rate has increased from 1.2 million ha at the beginning of the 1990's (FAO 1993) to 1.3 million ha, or 1.2% of the country's forest cover at the end of the 1990's (FAO 2001a). This increase is driven by industrial and logging operations, population pressure, transmigration programs, and expanding agriculture. Furthermore, the road network built for commercial logging, which provides access to forest areas, increases the fragmentation of the forest (Sunderlin 1999) and leads to an expansion of margin areas, consequently increasing the vulnerability of the forest (Skole and Tucker 1993). The stability of rainforest margins is a critical factor in the preservation of tropical forests. Thus, one of the most important challenges is to find solutions for stabilizing these margin areas. Agroforestry may offer economically viable windows of opportunity for the sustainable use of tropical forests (Wassmann and Vlek 2003).

Nitrogen (N) is one of the most important nutrients affecting crop yields. However, low levels of N are often a limiting factor especially in developing countries (Crews 1999). Natural or anthropogenic disturbances are associated with drastic reductions in biomass and loss of N from the system. In shifting land-use systems, for instance, N rapidly becomes one of the growth-limiting nutrients causing the abandonment of the land (Franco and de Faria 1997). Slashing and burning 3.5- and 7-year-old secondary forest in the Eastern Amazon resulted in a negative N balance of about 291 and 403 kg N ha^{-1}, respectively (Sommer et al. 2004). Furthermore, in more open bush vegetation and grassland, repeated burning volatilized between 56 and 90 % of the aboveground N pool (Kauffman et al. 1994). Sustained land productivity and high crop yields can only be maintained by replenishing the soil nutrients removed by harvested products or lost via erosion, leaching, runoff, or volatilization; the replenishment should provide at least an equivalent amount of inputs, either from fertilizer and manure, or from natural processes. Therefore, the strategy of closing the nutrient cycle at the farm level through integrated soil fertility management by returning

residue, fallowing fields and enhancing biological nitrogen fixation (BNF) in farming practices is essential for sustainability (Vlek et al. 1997).

In the tropics, cultivation of cacao (*Theobroma cacao* L.) with N_2 fixing trees (NFTs) such as Gliricidia (*Gliricidia sepium* (Jacq.) Walp) as shade trees has been practiced for generations. In this agroforestry system, NFTs are mainly used to reduce the heat stress of the cacao through the amelioration of the micro-climatic conditions (Beer at al. 1998). In addition, NFTs provide a possible solution for the restoration and maintenance of soil fertility, the reduction of soil erosion, and the maintenance of productivity for a longer period of time (Khanna 1998; Jose at al. 2000). Studies carried out in coffee and cacao plantations in Latin America showed that the aboveground N from NFTs varied from 60-340 kg N ha^{-1} yr^{-1} (Beer 1988). Roskoski and van Kessel (1985) reported N_2 fixation between 35-60 kg N ha^{-1} yr^{-1} by different NFTs in an unfertilized coffee and cacao plantation. Meanwhile, BNF by Gliricidia was estimated to range between 100 and 300 kg N ha^{-1} yr^{-1} (Khanna 1998; Franco and de Faria 1997; Sanginga et al. 1995). Thus, N_2 fixation by Gliricidia can play a major role for the input of N into the soil. However, shade trees also have physiological drawbacks, such as competition that reduces the production of the main crops. As a consequence, farmers often alter their cacao agroforestry system to "full-sun" cacao monoculture without NFTs. This practice leads to a short-term increase in cacao production, but in the long run it increases stress and the need for nutrients, especially N, and pesticides, and reduces the period of productivity (Beer et al. 1998; Siebert 2002). The farmers' lack of information regarding the amount of N_2 fixed by the NFTs and their contribution to the N economy of the cacao agroforestry systems is the main reason for this practice. This lack of information is not only due to the low economic value of shrub and leguminous trees compared to food grain legumes (Moufhe and Dakora 1999), but also to the fact that N_2 fixation by NFTs is difficult to measure (Boddey et al. 2000).

It is clear that BNF can play a major role in the restoration of soil fertility in agroecosystems. However, its role in a specific cacao agroforestry system where the NFTs such as Gliricidia are mainly used as shade trees remains unclear. Field research on this question is very limited and mainly based on the assumption that NFTs will fix N_2 from the atmosphere, thus increasing the availability of N to the system. However, there are still some open questions such as: Does Gliricidia play a major role in the

restoration of soil fertility in cacao agroforestry systems? How much N_2 is fixed by the Gliricidia annually? And what are the consequent implications for management practices (e.g. pruning and litterfall input)? In order to answer these questions, quantifying the proportion of N derived from atmospheric N_2 (%Ndfa) in the N balance of the cacao agroforestry system is crucial.

There are several methods for estimating N_2 fixation in NFTs such as the total N difference method (Gauthier et al. 1985; Ndoye and Dreyfus 1988), the acetylene reduction assay method (Roskoski 1981; Roskoski et al. 1982), or the ureide assay method (Herridge et al. 1994; Peoples et al. 1996). These methods, except the N different method that is direct criteria, are based on indirect, qualitative, yield-dependent criteria, and furthermore, not all NFTs carry ureides in their xylem sap. Therefore, the ^{15}N isotope method has become a widely used technique for estimating N_2 fixation in legumes, because it provides yield-independent and time-integrated estimates of %Ndfa (Chalk 1985; Shearer and Kohl 1986; Peoples et al. 1995; Boddey et al. 2000). The ^{15}N isotope method depends upon differences in isotopic composition of the sources of N available for plant growth, i.e., soil N, fertilizer N and atmospheric N_2 (Bergersen and Turner 1983). There are two main variations of the technique: One involves enrichment of soil N by addition of ^{15}N-enriched fertilizers (^{15}N enrichment method, ^{15}NEM), and the other makes use of the natural ^{15}N enrichment of available soil N (^{15}N natural abundance method, $^{15}NNAM$).

The ^{15}NEM is widely used and has found widespread acceptability for annual crops and herbaceous forage legumes. It has also been reviewed by Chalk (1985), Danso (1988), Witty et al. (1988) and Giller and Wilson (1991). The underlying assumption of the technique is that fixing and reference plants absorb the same relative amount of nitrogen of ^{15}N-enriched fertilizer from the soil; in theory the added enriched fertilizer must be homogeneously distributed vertically and horizontally over the rooting zone of the plants. Some reviewers point out that in a number of cases this assumption is violated, producing considerable errors in the estimation of BNF. This is especially true for natural conditions encountered in the field, when woody, deep-rooting perennials are involved extracting different (possibly non-labeled) pools of N (Danso et al. 1992; Parotta et al. 1994). Although some “ideal” reference plants have been defined in the literature (Witty 1983; Danso et al. 1992), it is difficult to choose an appropriate

reference plant to satisfy the requirements in the case of long-term studies with NFTs, especially in a permanent system such as cacao agroforestry, where only two or three plant species are available. This may also not be easily or consistently met when faced with a rapid change of ^{15}N enrichment in the soil both with time and depth (Chalk 1985; Giller and Wilson 1991; Danso et al. 1992).

The ^{15}NNAM is, on the other hand, seen as the most promising methodology for quantifying the contribution of N_2 fixation in natural systems (Boddey et al. 2000). It is based on the difference in $\delta^{15}N$ values (‰, between the two sources of N nutrition, soil-mineral N and atmospheric N_2 calculated as 1000 x (atom% ^{15}N sample – atom% ^{15}N reference) / atom% ^{15}N reference, with atom% ^{15}N reference at 0.3663 %. The accuracy of the estimates of N_2 fixation using this technique is influenced by the degree and uniformity of the $\delta^{15}N$ values in the plant-available soil N (Shearer and Kohl 1986; Gathumbi et al. 2002). In many cases, the variation of $\delta^{15}N$ values of total N in the soil is small and reasonably uniform and stable with time (Högberg 1997); therefore, the choice of a reference plant appears less critical. However, the $\delta^{15}N$ value of the plant-available soil N may vary spatially and temporally (Ledgard et al. 1984), which complicates the assessment of N_2 fixation by NFTs. Though the ^{15}N pool is not enriched artificially, isotopic discrimination is the main bottleneck in this method (Sutherland et al. 1993; Androsoff et al. 1995). A minimum of 2 ‰ $\delta^{15}N$ unit differences between the plant-available soil N (detected in reference plant) and atmospheric N_2 (detected in fixing plant) is recommended (Uncovich et al. 1994). Shearer and Kohl (1986) recommend a minimum 5-7 ‰ $\delta^{15}N$ value for plant-available soil N, which appears to be more adequate given the potential problem with spatial variability and isotopic discrimination. Gathumbi et al. (2002) suggest a >5 ‰ $\delta^{15}N$ value of plant-available soil N for tree-based fallow systems. Additionally, variation in the $\delta^{15}N$ value of the legumes under study and seasonal changes as well as differences between plant compartments might cause uncertainties, a fact widely recognized in the literature (Shearer et al. 1983; Bremer and van Kessel 1990; Sutherland et al. 1991; Unkovich et al. 1994; Pate et al. 1994; Sanginga et al. 1996).

A number of field and greenhouse studies have compared the ^{15}NEM and the ^{15}NNAM for estimating %Ndfa in different legume species under a variety of growing conditions. Both methods provide similar estimates of N_2 fixation (Bremer and van

Kessel 1990; Peoples et al. 1996; Cadisch et al. 2000). However, a good agreement does not always imply that both methods provide a correct estimate (Hamilton et al. 1993; Cadisch et al. 2000), as correlations between the two methods in individual estimates are poor (Androsoft et al. 1995; Stevenson et al. 1995). This is probably caused by a high spatial variability in controlling environmental variables (Boddey et al. 2000; Walley et al. 2001). In addition, a small standard error of the estimate does not always indicate that the estimate is accurate (Witty and Ritz 1984). Furthermore, most experiments on different methods report the overall average of the estimates and it remains unclear whether individual values are correlated or not.

Based on the problems discussed above, this study was conducted to test the hypotheses that

(1) Gliricidia can play a major role in cacao agroforestry systems not only as a shade tree but also for the restoration of soil N fertility;

(2) The ^{15}NNAM can be used successfully to estimate %Ndfa of Gliricidia in cacao agroforestry systems as an alternative to the ^{15}NEM, as long as appropriate precautions are taken.

Therefore, the objectives of this study are:

(I) to quantify the %Ndfa of Gliricidia in a cacao agroforestry system, and

(II) to compare ^{15}NEM and ^{15}NNAM in estimating the %Ndfa of Gliricidia in a cacao agroforestry system.

2 LITERATURE REVIEW

2.1 Biological nitrogen fixation

Frequently, subsistence farmers face the problem that the capacity of their soil to supply N declines rapidly once agriculture intensifies (Herridge et al. 1994). This condition is more often encountered in the tropics, where many soils are extremely fragile and give very poor yields after only a few years of cultivation without expensive fertilizer inputs. Soil erosion and further decline in soil nutrient status is often a consequence. Therefore, to conserve productivity and to achieve sustainable management, it is necessary to replenish nutrients that have been removed or lost from the soil. In the case of N, this can be achieved either by applying nitrogenous fertilizers or through BNF (Peoples et al. 1995). However, the working concept of sustainable agriculture for tropical developing countries aims to avoid the excessive use of mineral fertilizers, energy and pesticides. For that reason, and since atmospheric N_2 is a virtually inexhaustible source, and the energy used for BNF is 'free', the use of BNF is the most 'environmentally friendly' approach to supply N and organic matter to an agroecosystem (Bohlool et al. 1992; Danso et al. 1992). It also plays a role in reducing the production risk and in the management of the agriculture resource base (Giller and Cadisch 1995). In contrast to the optimistic view that soil-improving legumes may play a significant role in agricultural systems, there are also growing concerns that the use of soil-improving legumes is declining worldwide (Becker et al. 1995)

Galloway et al. (1995) estimated the total annual global bio-fixation in agroecosystems at around 40-48 million Mg N yr^{-1}, which represents almost half of the 90 million Mg N yr^{-1} from industrial fertilizer production (FAO 2001b), and approximately 20 % of all N available to the world's crops (Smil 2002). This means that a great effort would be necessary if BNF were to replace industrial fertilizers (Bumb 1995). On the other hand, with recent international emphasis on environmentally sustainable development and the use of renewable resources, greater attention will be given to the role of BNF in supplying N for agriculture (Peoples et al. 1995).

Biological nitrogen fixation, without doubt, improves the N status of soil. But this does not mean that legumes always contribute large amounts of N to the soils in which they grow. Perennial agroforestry systems are especially predestinated to provide

a more positive N balance and to minimize N losses to the environment (Peoples et al. 1995). Biological nitrogen fixation can also be controlled by manipulating various physical, environmental, nutritional, and biological factors (Hansen 1994) and may be more open to management (Khanna 1998). The use of inorganic N-fertilizer is on the other hand regulated by economic considerations, the fertilizer itself is generally utilized inefficiently by crops, and there is an increasing awareness of the environmental costs involved (Craswell and Godwin 1984; Peoples et al. 1995).

The fixation of the unavailable gaseous form of N_2 from the atmosphere into forms that higher plants can use (either NH_4^+ or NO_3^-) is mediated by: (1) bacteria in symbiotic relationship with vascular plants, (2) symbiosis between cyanobacteria and fungi (lichen) or plants, (3) free-living heterotrophic or autotrophic bacteria that are typically associated with soil or detrious, and (4) abiotic reactions in the atmosphere associated with lightning (Sprent and Sprent 1990). Biological nitrogen fixation by symbiosis of legumes with the bacteria *Rhizobium/Bradyrhizobium*, by rhizosphere associations (e.g., with the *Gramineae* or the *Ulmaceae*) and by free-living microorganisms in the phyllosphere and in the soil varies widely regarding their quantitative importance. Of those inputs, symbiotic N_2 fixation by legume-rhizobia association provides the largest inputs of N for agriculture (Peoples et al. 2002) and is an important component of the N cycle in agroecosystems.

2.1.1 Biological nitrogen fixation in agroforestry systems

In the conceptual development of ecologically sustainable production, systems that require minimal industrial inputs, such as agroforestry systems with legumes and non-legume crops, have raised much hope in recent years. The energy crisis will continue to increase the cost of chemically fixed nitrogen (Burrish 1999). Hardy (1980) estimated that 1-2 % of the world's fossil energy is used for fertilizer. Therefore, integrating trees, especially NFTs, into agroforestry systems can make a major contribution to sustainable agriculture (Giller and Wilson 1991; Cinnamani 1993; Peoples et al. 1995; Palm 1995). Nitrogen fixing trees are especially valued for their ability to grow in soils poor in N (Boddey et al. 1997; Bhatia et al. 2001).

The use of legumes as shade trees is one of the earliest examples of the use of NFTs in agroforestry (Giller 2001) despite the fact that in this system, NFTs are mainly

used to protect the main crops, e.g., cacao or coffee, from the 'full' sun, rather than as a source of N to improve soil fertility. Two factors regulate the amount of N_2 fixed by legumes: the amount of N accumulated during growth, and the proportion of N that is derived from symbiotic N_2 fixation (Peoples et al. 1997). Transfer of N from N_2-fixing plants to non-N_2-fixing plants in mixed stands can occur in various ways, i.e., aboveground processes influenced by the production of litter fall (or pruning), its nutrient content, and the rate of decomposition, and belowground processes influenced by the turnover of fine roots and nodules (Khanna 1998). On the other hand, competition between plants for light, space, nutrients, and water in many cases reduces the productivity of the main crops.

Little information is available on NTFs regarding their lower economic value compared to food grain legumes (Muofhe and Dakora 2000). The problems associated with BNF measurement are much more complex with NTFs than with annual crops, largely due to the size of trees and their perennial nature (Danso et al. 1992), which make an assessment of total biomass and N content difficult. Moreover, high variability may exist among isolines and provenances (Sanginga et al. 1992). Hence only few studies have been conducted on BNF in trees.

2.1.2 Gliricidia (*Gliricidia sepium* [Jacq.] Walp.)

Gliricidia, also named "madre de cacao" (Spanish for "mother of cacao") to describe its use as a cacao shade tree, is one of the most common multipurpose woody legumes throughout the tropics. It is a member of the sub-family *Papilionoideae* and of the tribe *Robinieae* (Lavin 1987). It is closely related to but not synonymous with a less common white flowered taxon, *Gliricidia maculata.* It is native to the Pacific coast of Central America and Mexico (Simons and Stewart 1998; Nygren et al. 2000). In Indonesia, especially in Sulawesi, most cacao agroforestry systems use Gliricidia as a shade tree due to its resistance to the defoliating psyllid (*Heterosphylla cubana*), which has devastated the NFT *Leucaena leucocephala* in cacao and coffee agroforestry systems in Sumatra (Swaminathan 1987).

Gliricidia residue is rich in N. With low concentrations of lignin and active polyphenol, it decomposes rapidly (Handayanto et al. 1994; Vanlauwe 1996); therefore, when used as a green manure or organic fertilizer, in a short time it contributes a large

amount of N available to the other plants. It has also been reported that decomposed litter of Gliricidia increases nutritional status, water holding capacity and bulk density of the soil (Rosecrance et al. 1992; Arachchi and Liyanage 1998). Nitrogen release from litterfall of Gliricidia trees may reach 20 kg N ha^{-1} yr^{-1} (Nygren et al. 2000). The nodule turnover may also reach 20 kg N ha^{-1} yr^{-1} (Nygren and Cruz 1998). Gliricidia, with a deep-rooting system, has shown the ability to loosen hard soils (Toky and Bisht 1992).

Table 2.1 shows the %Ndfa of Gliricidia estimated with different methods. The %Ndfa estimate of Gliricidia ranges from 37-55 % in hedgerow trees to 41-43 % in alley cropping and 49-87 % in monoculture. The input of atmospheric N_2 by Gliricidia varies considerably depending on the method used to estimate BNF, the age and the population of the trees and the management practices. Studies carried out in coffee and cacao plantations in Latin America with 120-560 leguminous trees ha^{-1} show that the aboveground N transfer input varied from 3-14 Mg ha^{-1} yr^{-1} of dry matter containing 60-340 kg N $ha^{-1}yr^{-1}$ (Beer, 1988). Roskoski and van Kessel (1985), applying the acetylene reduction assay to determine the input of atmospheric N_2 in an unfertilized coffee and cacao plantation by the shade trees *Inga junicuil, Gliricidia sepium,* or *Erythrina poeppigiana,* report N_2 fixation between 35 and 60 kg N ha^{-1} yr^{-1}. Use of the same technique in field monoculture resulted in estimates of only 13 kg N ha^{-1} yr^{-1} (Roskoski et al. 1982); estimates using isotope dilution methods showed that Gliricidia contributed 86-309 kg N ha^{-1} yr^{-1} in alley cropping in the Philippines (Ladha et al. 1993) and 70-274 kg N ha^{-1} yr^{-1} in field monoculture in Australia (Peoples et al. 1996). Though BNF by Gliricidia varied depending on the management and method used, the results show that BNF by Gliricidia can play a significant role in agricultural systems.

Table 2.1: Biological nitrogen fixation by Gliricidia estimated with different methods

Management	Estimated BNF		Method	Reference
	%Ndfa	kg N ha^{-1} yr^{-1}		
Field, monoculture	72	108	TND	Liya et al. (1990)
Cacao and coffee plantation	nd	60-340	TND	Beer (1988)
Cacao and coffee plantation	nd	35-60	ARA	Roskoski and van Kessel (1985)
Field, monoculture	nd	13	ARA	Roskoski et al. (1982)
Field, monoculture	41-43	12-23	^{15}NEM	Sanginga et al. (1994)
Alley cropping	52-64	86-309	^{15}NNAM	Ladha et al. (1993)
Hedgerow trees	37	18	^{15}NNAM	Hairiah et al. (2000)
Hedgerow trees	55	26	^{15}NEM	Hairiah et al. (2000)
Monoculture trees	56-89	70-274	^{15}NNAM	Peoples et al. (1996)
Monoculture trees	49-87	70-274	^{15}NEM	Peoples et al. (1996)
Alley cropping	55	166	^{15}NEM	Liyanage et al. (1994)

BNF is biological nitrogen fixation; %Ndfa is the proportion of N derived from atmospheric N_2; TND is total N different method; ARA is acetylene reduction assay; ^{15}NEM is ^{15}N enrichment method; ^{15}NNAM is ^{15}N natural abundance method; nd = no data

2.2 Methods for quantifying biological nitrogen fixation

The potential for BNF by legumes can be assessed in a relatively simple way by determining the active nodulation of the roots of these plants and scoring the nodulation taking into account nodule number, size, pigmentation and distribution (Sylvester-Bradley 1980; Sprent et al. 1996). Nevertheless, N_2 fixation cannot be quantified with this method. Thus, other methods were developed to assess N_2 fixation contribution to the nutrition of leguminous plants. The commonly used methods for measuring BNF are the total N difference, xylem solute technique, acetylene reduction assay, and ^{15}N isotope techniques. In spite of the various advantages and disadvantages of these methods, the ^{15}N isotope techniques have been widely adopted for estimating N_2 fixation in legumes, principally because they provide yield-independent and time-integrated estimates of %Ndfa (Chalk 1985; Shearer and Kohl 1986; Peoples et al. 1995; Boddey et al. 2000).

The ^{15}N isotope techniques depend upon differences in the isotopic composition of sources of N available for plant growth, i.e., soil N, fertilizer N and atmospheric N_2 (Bergersen and Turner 1983), which may arise from the addition of ^{15}N-enriched labeled fertilizer or ^{15}N-depleted materials to the soil (^{15}NEM), or from the low natural enrichment of ^{15}N in the soil (^{15}NNAM). Both methods are based on the same principle, except that in the ^{15}NNAM, it is the small differences that occur between

atmospheric N_2 and the natural ^{15}N enrichment of the soil, due to the isotopic discrimination processes during N transformations that is utilized to estimate N_2 fixation (Shearer and Kohl 1986; Peoples et al. 1997; Boddey et al. 2000).

The ^{15}NEM and ^{15}NNAM rely on the use of a reference plant to assess the relative availability of soil N over the growing period, which is assumed to be the same for both fixing and reference plants. Therefore, the choice of the reference plant can have a great effect on the estimation of N_2 fixation (Ledgard et al. 1985; Evans et al. 1987; Witty et al. 1988; Danso et al. 1992; Boddey et al. 2000). It is generally accepted that the reference plant species should be similar to the fixing plant in terms of phenology, rooting profile and pattern of utilization of the soil N pool. The reference plant is generally assumed to be the ultimate source of error if the temporal and spatial distribution of the ^{15}N in the soil is non-uniform (Fried et al. 1983; Domenach and Corman 1984; Shearer and Kohl 1986; Witty et al. 1988; Boddey et al. 1995; Chalk and Ladha 1999). Non-nodulating isolines of legumes have therefore been considered suitable reference plants for measuring the BNF of their nodulated "counterpart" (Cadisch et al. 2000). However, a non-nodulating legume is rarely available, especially for fixing trees; in addition, there is also some evidence that non-nodulating legumes as reference plants may not always be ideal (Ruchel et al. 1979; Rennie 1982). On the other hand, in many cases the reference plant that has been recommended as suitable for a given legume in one situation may not be suitable in another, since the so-called "appropriate" reference plant may be site- and season-specific (Boddey et al. 1995).

2.2.1 Nitrogen-15 enrichment method (^{15}NEM)

The ^{15}NEM (e.g., McAuliffe et al. 1958; Chalk 1985) has been widely used in agricultural systems and has found widespread acceptability for annual crops and herbaceous forage legumes. Unfortunately, the application of the technique to quantify the contribution of %Ndfa by fixing perennial plants, especially NFTs, poses problems dealing with (1) the long-term, perennial nature of growth and seasonal changes in the pattern of N assimilation, (2) the large plant to plant variation in growth and N demand, and (3) the difficulties in the accurate quantification of the large amounts of standing biomass and N produced by NFTs (Shearer and Kohl 1988; Danso et al. 1992; Boddey et al. 2000; Peoples et al. 2001).

The ^{15}NEM requires the application of a small dose of the ^{15}N-enriched (labeled) fertilizer to the soil. The objective of this enrichment is to artificially increase the difference in ^{15}N content between the N sources in the soil and atmospheric N_2. The greater the ^{15}N enrichment of the plant-available soil N pool that can be achieved, the greater the accuracy of subsequent calculations (Peoples et al. 2001). Based on the assumption that a reference plant takes up a similar proportion of soil fertilizer-^{15}N to the fixing plant, the %Ndfa can be calculated. The advantage of this method is that it provides a yield-independent and time-integrated %Ndfa estimate. However, the validity of this method can only be reliably tested when the uptake of labeled N by the fixing plant equals the uptake by the reference plant (Chalk 1985; Ledgard et al. 1985), which in fact means that the added enriched fertilizer must be homogeneously distributed vertically and horizontally over the rooting zone of the plants. This is very difficult to achieve in the field. Additionally, despite the high cost of ^{15}N fertilizer, the rapid decline in ^{15}N enrichment of plant-available soil N with time (Witty 1983) is another disadvantage of this method. Finally, the large variation in rooting depth and size, the long growth period of trees, and the non-uniform vertical distribution of applied ^{15}N makes it difficult to find matching reference trees (Danso et al. 1992; Hairiah et al. 2000). In short, the selection of an appropriate reference plant and the technique of adding labeled ^{15}N are particularly critical in ^{15}NEM.

Reference plant

Asynchrony of mineral uptake by fixing and reference plants due to different growth patterns, rapid decline of ^{15}N from applied fertilizer (Witty 1983; Rennie and Rennie 1983; Chalk 1985; Doughton et al. 1995), different root distribution and variable ^{15}N concentration in soil mineral N, especially at low values of %Ndfa (Hardarson et al. 1988; Danso et al. 1992; Boddey et al. 1995) have been identified as sources of error in quantifying the %Ndfa of fixing plants. The reference plant is, therefore, considered to be suitable if it does not fix N_2 and has the following characteristics in common with the fixing plant: rooting zone, relative N uptake pattern, and growth duration (Witty 1983; Danso 1988). This does not necessarily mean that plants should have equal rooting depths (Danso et al. 1992), but since the ^{15}N content of plant-available soil N varies with depth and time, it is crucial to select a reference plant whose time course of N

uptake has the same pattern as the fixing plant (Fried et al. 1983; Shearer and Kohl 1988; Danso et al. 1992).

Uninoculated host trees have been used as reference plants (Gauthier et al. 1985; Ndoye and Dreyfus 1988), but cross contamination may occur. Non-nodulating isolines, as used in annual crops, may be the best alternative (Cadisch et al. 2000), but not all legumes have non-nodulating isolines, especially NFTs. Rennie (1986) suggests that choosing the most appropriate reference plant is probably more important than the problem of non-uniform distribution of the applied ^{15}N in the soil.

Application of ^{15}N fertilizer

There are several factors that influence the utilization of ^{15}N by fixing and reference plants, e.g., the chemical and physical form of labeled fertilizer, and the time, rate, and the method of the application of the ^{15}N-labeled fertilizer (Chalk 1985). Several procedures for uniform application of ^{15}N-enriched material to the soil have been employed, such as the addition of solution or solid forms, or application in cellulolytic compounds, in plant residue grown on enriched soil, or of already enriched soil (Kohl and Shearer 1981; Witty 1983; Fried et al. 1983; Witty and Ritz 1984; van Kessel and Nakao 1986).

A single addition of ^{15}N to the soil in a soluble fertilizer is the most common approach. However, this practice results in a rapid decline of the ^{15}N enrichment, as the soil mineral N pool is continuously replenished by unlabeled N from the mineralization of soil organic matter (Boddey et al. 1995). Thus, if the reference and fixing plants have different temporal patterns of soil N uptake, they will tap different levels of ^{15}N enrichment in the soil-derived N. The greater the changes in the enrichment of soil mineral N with time, the greater will be the error in the estimate of BNF due to mismatching of the uptake patterns of reference and fixing plants (Witty 1983; Chalk 1985; Rennie 1986; Danso 1988). This is especially true for the natural conditions encountered in the field, when woody, deep-rooting perennials are involved that extract different (probably non-labeled) pools of N (Parotta et al. 1994; Danso et al. 1992). For this reason, Baker et al. (1990) recommend the trenching of the perimeter of each replication block and installing a multi-layer plastic film or, alternatively, frequent addition of ^{15}N fertilizer, which can possibly reduce the ^{15}N label differences in the root

zones (Danso 1988). In addition, enriching a soil with ^{15}N fertilizer through injection of mineral N fluid is recommended instead of spraying or banding methods (Rennie 1986). Furthermore, since high mineral N concentrations may inhibit N_2 fixation (Chalk 1985), the amount of N applied should be small enough to not significantly suppress BNF.

2.2.2 Nitrogen-15 natural abundance method (^{15}NNAM)

The principle of the ^{15}NNAM is the same as that of the ^{15}NEM, except that ^{15}N-enriched fertilizer is not applied to the soil. The technique, which holds most promise to quantify the contributions of N_2 fixation to trees in the field (Boddey et al. 2000) is based on the naturally occurring difference between the ^{15}N abundance of the two sources of N-nutrition, soil mineral N and atmospheric N_2. As the ^{15}N isotope is slightly heavier than the ^{14}N, compounds containing ^{15}N tend to react more slowly, particularly in reactions that lead to gaseous losses of N from the soil. The net effect is that the soil, over a long period of time, becomes slightly enriched with ^{15}N (Peoples et al. 1989; Giller and Wilson 1991; Yoneyama et al. 1993) (Figure 2.1). Calculation of %Ndfa using the ^{15}NNAM requires that both the ^{15}N natural abundance of the N derived from BNF and that derived from the soil by the target fixing plants be determined (Shearer and Kohl 1986; Boddey et al. 2000). Differences in the enrichment of fixing and reference plants reflect the dependence of the plants on atmospheric N_2 and are used to calculate N_2 fixation (Shearer and Kohl 1986; Giller and Wilson 1991; Peoples et al. 1997).

The ^{15}NNAM has several advantages: (1) the fairly stable $\delta^{15}N$ value in the soil with time results in lower errors in the determination of BNF, (2) no addition of costly ^{15}N fertilizer is required; for trees this could involve substantial savings, (3) no disturbance of the soil during incorporation of ^{15}N fertilizer, and (4) estimation of %Ndfa is integrated over the entire growing season (Shearer and Kohl 1986; Danso et al. 1992; Boddey et al. 2000). However, the drawback is that small isotope fractionations or small variability among sites, species or plant parts can cause significant errors (Shearer and Kohl 1986). There are also some difficulties in implementing this method, not only in natural ecosystems but also in agroforestry and plantation systems. These are related to: (1) the level of spatial and temporal variability of the $\delta^{15}N$ value of the plant-available soil N, (2) the uptake of different sources of N with different $\delta^{15}N$ values, (3) isotopic fractionation occurring during N_2 fixation, and

(4) different $\delta^{15}N$ values in soil N under fixing and reference plants over time as a result of leaf litter and senescent roots (Shearer and Kohl 1986; Hansen and Pate 1987; Pate et al. 1993; van Kassel et al. 1994; Högberg 1997).

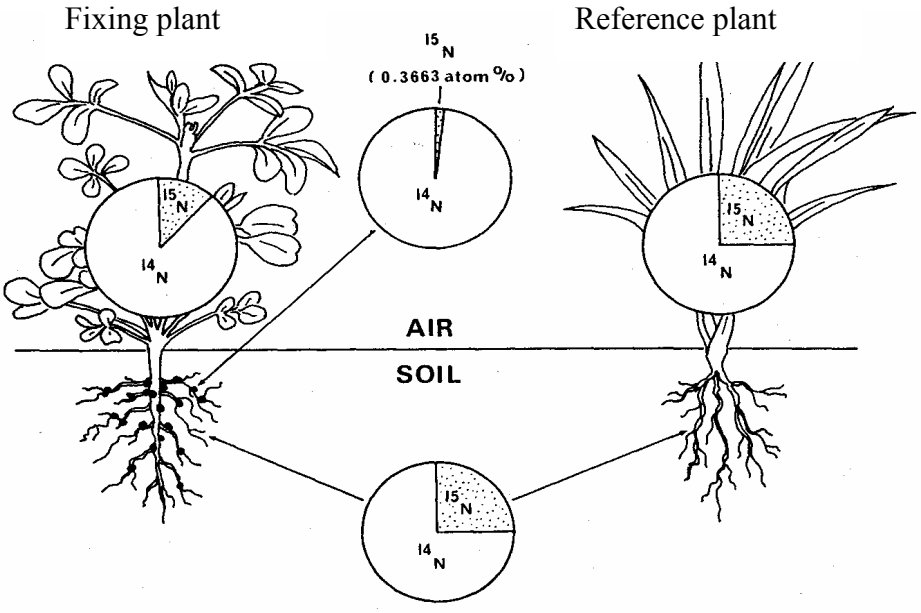

Figure 2.1: Principles of the ^{15}N natural abundance method (Source: Peoples et al. 1989)

Reference plant

The accuracy of ^{15}NNAM also depends upon the choice of the right reference plant. A high variability of the $\delta^{15}N$ value of the reference plant encountered in the field may lead to differences in %Ndfa. Thielen-Klinge (1997), for instance, encountered a difference of up to 20 % depending on the chosen species. To assess the uncertainty involved in the BNF estimates, a range of reference plants is advisable; parallel glasshouse studies with ^{15}N enrichment for a better identification of suitable reference plants are an alternative (Boddey et al. 2000). Plants dependent on soil N often have $\delta^{15}N$ values close to the $\delta^{15}N$ value of soil organic matter (SOM), although under both temperate and tropical conditions, reference plants can apparently still have lower $\delta^{15}N$ values than in the SOM (Högberg and Alexander 1995; Domenach et al. 1989). Therefore, for reference plants, it is more reasonable to use the $\delta^{15}N$ value of a plant whose sole source of N is soil-derived N than to rely on the $\delta^{15}N$ value of total soil N or NO_3^- mineralized under laboratory conditions (Shearer and Kohl 1986). Differences in

$\delta^{15}N$ values between reference plants are less influenced by soil depth than by differences between species (Gathumbi et al. 2002). Thus, the choice of an appropriate reference plant is critical even if the soil $\delta^{15}N$ value is relatively uniform with depth. Pate et al. (1994) report that the enrichment of $\delta^{15}N$ can differ considerably in reference plants growing at the same site. Boddey et al. (2000) and Gathumbi et al. (2002) suggest that the ^{15}NNAM is a useful tool for estimating the %Ndfa by field-grown herbaceous and woody legumes in soil with sufficient and relatively uniform background $\delta^{15}N$ values if appropriate, or a range of reference plants are used.

Plant $\delta^{15}N$ signature

Another important factor to be considered in ^{15}NNAM is the isotopic fractionation, which may occur during N_2 fixation, assimilation, protein synthesis, transport, and translocation of fixed N. In the tissues of fixing plants, N becomes diluted by the lower $\delta^{15}N$ value of fixed atmospheric N_2. This can be a result of isotopic fractionation and discrimination in both soil N transformations and plant uptake (Shearer and Kohl 1986) leading to variations in the $\delta^{15}N$ values of plant parts (Yoneyama et al. 1986; Cadisch et al. 1993). This is influenced by environmental factors such as the availability of water and nutrients (Ledgard and Peoples 1988) as well as the degree of infection, the type of rhyzobium strain and the involved mycorrhizal symbionts (Steele et al. 1983; Pate et al. 1993; Cadisch et al. 1993; Högberg and Alexander 1995). Branches or twigs of plants generally have lower $\delta^{15}N$ values than leaves (Shearer and Kohl 1986; Virginia et al. 1989). Furthermore, even in the same tissue, the $\delta^{15}N$ value often changes during growth and development (Boddey et al. 2000). The validity of using the $\delta^{15}N$ value on a particular organ to estimate BNF on a whole-tree basis is, therefore, questionable. However, leaves are the most convenient and valid samples for BNF estimations as they represent the largest single N component. In addition, the difference between their $\delta^{15}N$ value and that of other components is not large. Nevertheless, it is suggested that at least a preemptory survey should be made of different tissues (Boddey et al. 2000).

Soil $\delta^{15}N$ signature

The size and nature of the active N pool in soil is controlled by external input into the system, internal cycling by mineralization and immobilization, and losses through volatilization, denitrification, leaching or export of plant products (Kerley and Jarvis 1999). In general, soil N is more abundant in ^{15}N than atmospheric N_2, due to NH_4^+ assimilation and nitrification processes taking place simultaneously in the soil. With the latter having greater isotopic fractionation, the resulting nitrate is normally depleted in ^{15}N, and the N assimilated by the soil microbes is enriched. The microbial N is later deposited as recalcitrant organic N and nitrate leached or taken up by plants leading to overall enrichment of the soil N pool. Although exceptions were observed, the $\delta^{15}N$ value of soil was shown to vary from -6 to +16 ‰ (Shearer and Kohl 1986). Reference plants, whose primary source of N was soil-derived N, would be expected to have higher $\delta^{15}N$ values than fixing plants, which take N from both the atmospheric N_2 and the soil (Shearer and Kohl 1986; Peoples et al. 1989).

The $\delta^{15}N$ value of total soil N in the soil N pool may vary with depth (Ledgard et al. 1984), which can be problematical when assessing N_2 fixation in deep-rooting trees. The $\delta^{15}N$ values of total soil N can increase with soil depth, but the extractable soil mineral $\delta^{15}N$ signature may be more uniform with depth (Ledgard et al. 1984; Cadisch et al. 2000). It has also been reported that the $\delta^{15}N$ value of plant-available soil N (as detected in reference plants grown in soil taken from different depths) is not significantly different with depth (Ladha et al. 1993; Gathumbi et al. 2002). However, Koba et al. (1998) report that the $\delta^{15}N$ values of total N, NH_4^+-N and NO_3^--N increase with depth. Lower $\delta^{15}N$ values of the total soil N in the top layer are probably caused by plant N litter, which tends to show lower $\delta^{15}N$ values than the $\delta^{15}N$ values of the soil-N pool from which the plants derived their N (Bremer and van Kessel 1990). In agroforestry systems, the $\delta^{15}N$ value of plant-available soil N is apparently stable during the cropping season (Herridge et al. 1990) and tends to be more enriched in $\delta^{15}N$ than in undisturbed ecosystems and forests (Boddey et al. 2000). Shearer and Kohl (1986) recommend a minimal value of 5-7 ‰ $\delta^{15}N$ for plant-available soil N for the ^{15}NNAM, and Gathumbi et al. (2002) suggest a greater than 5 ‰ value for use in tree-based fallow systems.

Nitrogen-15 discrimination (*B*-value)

The *B*-value is usually determined as the $\delta^{15}N$ value of the fixing plant grown with atmospheric N_2 as the sole N source (Bergersen and Turner 1983). The $\delta^{15}N$ value is very close to that of atmospheric N_2, usually within 2 ‰ (Steele et al. 1983) and appears to range between -2.0 and +1.0 ‰ (Boddey et al. 2000). Nodules of most legume species are usually found to be more enriched in ^{15}N than other plant parts, indicating that most of the N present in the nodules is derived from N_2 fixation and not through reallocation of N from non-nodular tissue (van Kessel and Nakao 1986). The *B*-values are affected by the rhizobial strains used for inoculation (Steele et al. 1983) and plant parts used (Yoneyama et al. 1986). Therefore, the same rhizobial strain should be used for legumes grown in the N-free media and in the field. Table 2.2 shows some results of ^{15}N discrimination in legume trees.

Table 2.2: Nitrogen-15 discrimination in legume shrubs and trees

Species	$\delta^{15}N$ value of plant parts (‰)		Reference
	Shoot	Nodules	
Albizia lebbeck[c]	+7.10	+13.10	1
Asphalatus linearis	-2.0	n.d.	2
Calliandra	-1.29	+10.05	3
Codariocalyx	-1.83	+4.53	3
Dalea mollissimai[b]	-1.3	+2.5	4
Dalea schotii[a]	-2.0	+6.3	4
Flemingia congesta	-1.32	n.d.	5
Gliricidia sepium	-1,11	n.d.	5
Gliricidia sepium	-1.45	+4.78	3, 6
Leucaena luecocephala	-0.34	+10.11	3
Medicago sativa[b]	+0.60	n.d.	7
Sesbania grandifolia	-0.47	+12.03	3
Trifolium subteraneuma[b]	+0.96	n.d.	7

[a] Corrected for the $\delta^{15}N$ value of non-inoculated (non-nodulated) plants; [b] Entire plants; [c]Recalculated from $\%^{15}N$; n.d. = not determined; (1) van Kessel and Nakao (1986); (2) Moufa and Dakora (1999); (3) Peoples et al. (2001); (4) Shearer and Kohl (1986); (5) Hairiah et al. (2000); (6) Ladha et al. (1993); (7) Ledgard et al. (1985)

2.2.3 Comparison of ^{15}NEM and ^{15}NNAM

In a number of field and greenhouse studies, ^{15}NEM and ^{15}NNAM for estimating %Ndfa in different legume species under a variety of growing conditions have been compared (Appendix 1). In general, comparisons of %Ndfa estimated using ^{15}NEM and ^{15}NNAM yield similar mean values for %Ndfa (Bremer and van Kessel 1990; Androsoft

et al. 1995; Stevenson et al. 1995; Peoples et al. 1996; Cadisch et al. 2000). However, poor agreement between the two methods has also been reported (Hamilton et al. 1993; Androsoft et al. 1995; Stevenson et al. 1995; Walley et al. 2001). In addition, good agreement between the mean estimates of %Ndfa of fixing trees does not result in a good agreement between individual (paired-samples) estimates (Androsoft et al. 1995; Stevenson et al. 1995). Furthermore, a good agreement between the methods and a small standard error on the %Ndfa estimate does not necessarily imply that both methods provide correct estimates (Witty and Ritz 1984; Bremer and van Kessel 1990; Cadisch et al. 2000).

According to Handley and Scrimgeour (1997), the ^{15}NEM and ^{15}NNAM essentially reflect different processes. They argue that in ^{15}NNAM, large fractionation of δ^{15}N values in samples due to a variety of biotic and abiotic processes cannot be used as a tracer of N from source to sink, and it can, therefore, not be inferred that differences in δ^{15}N values between fixing and reference plants are caused primarily by BNF. In contrast, when using ^{15}NEM, the relative impact of isotope fractionation on the ^{15}N signature of the plant is insignificant. However, Boddey et al. (2000) and Walley et al. (2001) state that poor agreement between both methods may be caused by high spatial variability in the controlling environmental variables, and that the two approaches are essentially measuring the same process. Therefore, using two or more independent methods of measurement based on different principles is advisable in field N_2 fixation studies whenever possible (Peoples et al. 1997; Witty and Ritz 1984).

3 MATERIALS AND METHODS

3.1 Study area

The study was carried out from July 2002 until June 2003 in close cooperation with the interdisciplinary research project on "Stability of Tropical Rainforest Margins in Indonesia – STORMA" funded by the German Research Council (Deutsche Forschungsgemeinschaft – DFG, Sonderforschungsbereich – SFB 552).

The study area is located around the Lore Lindu National Park (Taman Nasional Lore Lindu, TNLL) in Central Sulawesi, Indonesia, at 01°05`- 01°54` southern latitude and 119°54`- 120°19` eastern longitude (Figure 3.1). According to the STORMA sub-project A3 "Village survey and GIS data" (Maertens et al. 2002), the total study area covers more than 700,000 ha (31 % inside TNLL and 69 % outside TNLL).

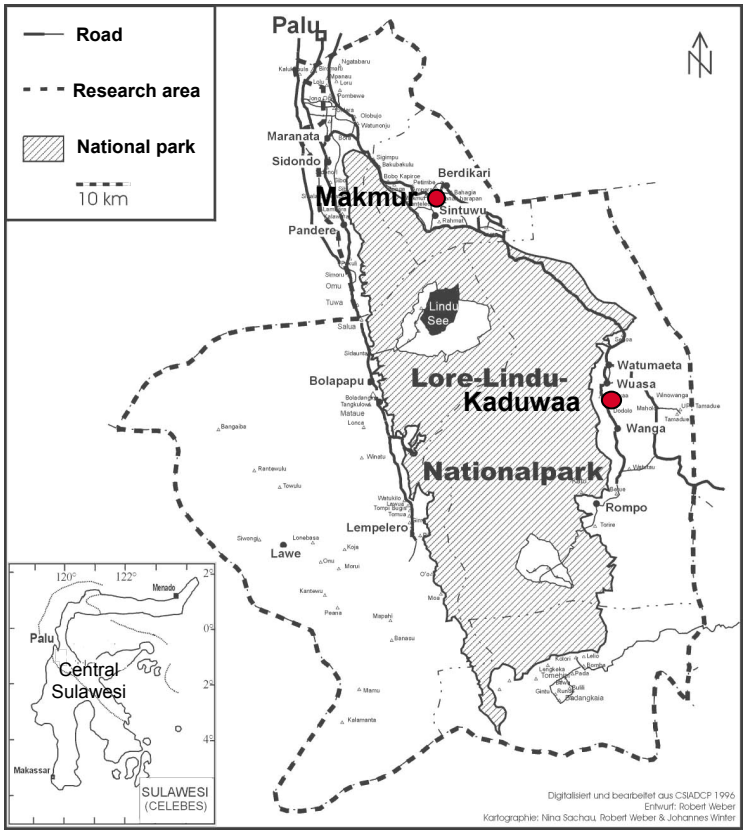

Figure 3.1: Study area around the Lore Lindu National Park (shaded area) (Source: STORMA [http://www.storma.de])

According to the preliminary survey and geological map (Sulawesi 2114, 1:250,000), crystalline and metamorphic rocks (granites, granodiorites, quartzites, crystalline slates and phyllites) are the source material in the mountains, tertiary sedimentary material (sandstones, marlacious and conglomerate rocks) occur in the valleys, and colluvial material is found in the downslope and foothill areas. Depending on the above parent materials and position, predominating soils (USDA classification) are Eutropepts, Tropudults or Dystropepts characterized by low soil organic matter (SOM) content and cation exchange capacity (CEC), high Al saturation and low plant-available P content (STORMA [http://www.storma.de]). The climate in the area shows high variability. Precipitation ranges from 500 to 2500 mm yr^{-1} depending on the altitude. Mean temperatures range from 10-12 °C in the high altitudes to about 25-26 °C in the coastal region (STORMA [http://www.storma.de]).

The land-use system is dominated by paddy rice in the valleys and alluvial plains, and agroforestry systems (cacao and/or coffee), annual crops (maize, cassava, beans, land rice), vegetables (tomatoes, cabbage, carrot, onion, chilli), and home gardens in the uplands. In the agroforestry system, Gliricidia is mainly used as a shade tree for cacao and coffee. A few farmers also use *Erythrina fusca* and *E. subumbrane* as shade trees. Crops such as chili, vanilla, pumpkin, ginger, banana, nut trees, avocado, and jackfruit are often planted in mixed stands with cacao and coffee. Many farmers have lately changed their traditional agroforestry system to a "full-sun" cacao system, cutting all the shade trees in order to increase the cacao production. In the short term, the practice proves to be more productive, but in the long run the impact is still an open question in terms of the period of productivity of the cacao and the balance of necessary nutrients (especially N) and pesticides compared to the traditional systems.

The agroforestry systems have mainly been converted from primary forest after two or three years of cultivating annual crops and vegetables, and only a small proportion from fallow secondary forest and grassland (then mostly dominated by *Imperata cylindrica*). Land preparation is done by cutting and burning the dry biomass and followed one year after the establishment of NFTs by the planting of cacao or coffee between the shade trees. The soil is left undisturbed. The density of cacao and shade trees in the cacao agroforestry system as practiced by the farmers varies. Cacao density is mostly around 1100-1250 trees ha^{-1} at a 2 m x 4 m or 3 m x 3 m spacing. The

density of the shade trees varies according to the age of the cacao and farmer's practice. In young cacao, the legume spacing is 2 m x 2 m (2500 trees ha^{-1}) which is thinned to 4 m x 4 m (625 trees) when the cacao is 4-5 years old. The farmers generally plant local cacao varieties. Weeding is done manually three to four times a year. The main crops and shade trees are pruned once or twice a year. The biomass is left to decompose in the field, except for the big branches, which are used as planting material for new shade trees or as fences. Fertilizer is rarely used by resource-poor farmers, but in some cases, mainly in cacao monoculture, 100-150 kg urea ha^{-1} yr^{-1}, 50-100 kg Triple Super Phosphate ha^{-1} yr^{-1} and 30-60 kg KCl ha^{-1} yr^{-1} are used. Cacao is harvested frequently, whenever the cacao pod has just opened; only the beans are removed.

3.1.1 Site description

The experiment was carried out in Kaduwaa, Lore Utara District and Makmur, Palolo District, Central Sulawesi, Indonesia. Kaduwaa is located at 01°26'361'' southern latitude and 120°18'469'' eastern longitude at 1100 m asl (above sea level), and Makmur at 01°07'26.7'' southern latitude and 120°05'01.1'' eastern longitude at 550 m asl (Figure 3.1). The Kaduwaa site was formerly under forest cover and had been planted for three years with maize and vegetables before being converted into a cacao agroforestry system. In Makmur, after forest clearing, the site was used for five years for maize and other upland crops such as banana, chili and vegetables. The following ten years, the farming system comprised small, irrigated paddy fields. Due to an inadequate water supply, these were subsequently converted into the cacao agroforestry system.

At both sites, at the beginning of the experiment in 2002, the cacao trees were around 7 years old, with 8- and 8.5- year-old Gliricidia shade trees. At the Kaduwaa site, the farmer intercropped coffee between the Gliricidia and cacao rows (Figure 3.2). At the Makmur site, farmers grew vanilla, for which Gliricidia was used as the climbing tree (Figure 3.3). On both sites, a local cacao variety was planted.

Figure 3.2: Cacao agroforestry system in Kaduwaa

Figure 3.3: Cacao agroforestry system in Makmur

3.1.2 Soil

The soil texture was determined using the pipette method. Total organic C and N were determined with a C&N analyzer (dry combustion, Heraeus vario EL). Available P was extracted with the CAL-method (Ca-acetate-, Ca-lactate-solution, Schüller 1969). Exchangeable cations (K^+, Na^+, Ca^{++}, Mg^{++}, H^+ and ECEC) were extracted with $BaCl_2$ (Mehlich, 1953), and Al^{3+} was calculated by subtracting the ECEC with the sum of base cations plus H^+. The soil was also analyzed for Fe, Mn, and Zn using Atomic Absorption Spectrophotometry (AAS). In addition, bulk density was measured at each depth (three repetitions) with undisturbed samples (100-cm^3 steel cylinder).

Generally, the soils in both study sites were sandy loam Typic Dystrudepths. Dechert (2003) also found Inceptisols with parent material of sandy alluvial sediment nearby the study sites. The physical and chemical characteristics of the soil in Kaduwaa and Makmur to the depth 150 cm are shown in Table 3.1 and 3.2.

Table 3.1: Physical and chemical characteristics of the soil in Kaduwaa (0-150 cm)

Parameters	Soil depth (cm)				
	0-10	10-30	30-50	50-100	100-150
Bulk density (g cm^{-3})[1]	1.1 (0.0)	1.2 (0.1)	1.3 (0.1)	1.4 (0.1)	1.3 (0.1)
Sand (%)[2]	55.6 (1.4)	52.2 (1.3)	60.6 (1.4)	64.4 (2.9)	57.9 (1.9)
Silt (%)[2]	26.0 (0.9)	28.6 (0.6)	28.4 (2.5)	29.7 (1.0)	32.5 (1.1)
Clay (%)[2]	18.4 (0.6)	18.9 (1.0)	11.0 (3.0)	5.8 (3.8)	9.5 (2.0)
pH (H_2O)	5.6 (0.2)	5.5 (0.2)	5.3 (0.1)	5.1 (0.0)	5.1 (0.1)
pH (KCl 0.5*M*)	4.6 (0.0)	4.4 (0.1)	4.2 (0.1)	3.8 (0.0)	3.8 (0.2)
C (g 100 g^{-1})[3]	2.2 (0.2)	1.1 (0.2)	0.4 (0.1)	0.3 (0.0)	0.2 (0.0)
N (g 100 g^{-1})[3]	0.18 (0.01)	0.11 (0.02)	0.03 (0.00)	0.02 (0.01)	0.01 (0.00)
C/N	11.9 (0.9)	10.7 (0.3)	13.9 (2.2)	16.4 (1.5)	21.4 (0.8)
Available P (mg kg^{-1})[4]	3.5 (0.7)	3.0 (1.5)	0.5 (0.2)	0.8 (0.1)	0.5 (0.2)
K^+ ($cmol_c$ kg^{-1})[5]	0.25 (0.05)	0.10 (0.01)	0.05 (0.01)	0.06 (0.03)	0.08 (0.03)
Na^+ ($cmol_c$ kg^{-1})[5]	0.04 (0.00)	0.04 (0.00)	0.05 (0.01)	0.07 (0.01)	0.07 (0.01)
Ca^{++} ($cmol_c$ kg^{-1})[5]	7.2 (0.5)	5.5 (0.8)	4.2 (0.3)	2.9 (0.1)	2.7 (0.2)
Mg^{++} ($cmol_c$ kg^{-1})[5]	0.8 (0.1)	0.4 (0.2)	0.3 (0.1)	0.4 (0.1)	0.5 (0.1)
Al^{+++} ($cmol_c$ kg^{-1})[6]	14.7 (0.8)	12.2 (1.2)	11.1 (0.9)	10.8 (0.1)	11.2 (0.1)
ECEC ($cmol_c$ kg^{-1})[5]	22.9 (0.8)	18.3 (2.0)	15.8 (1.0)	14.2 (0.1)	14.6 (1.0)
Base saturation (%)[7]	36.2 (2.6)	33.2 (2.4)	29.4 (2.0)	24.4 (0.1)	23.0 (1.7)
Fe (mg kg^{-1})[8]	4.6 (0.7)	3.3 (0.6)	n.d.	n.d.	n.d.
Zn (mg kg^{-1})[8]	0.9 (0.4)	0.1 (0.1)	n.d.	n.d.	n.d.
Mn (mg kg^{-1})[8]	19.4 (2.0)	14.2 (1.5)	n.d.	n.d.	n.d.

Soil analysis methods: 1) gravimetric; 2) pipette; 3) dry combustion; 4) CAL-method; 5) Mehlich; 6) calculated; 7) calculated; 8) NH_4OAc; Value in parentheses represents standard error of the means; n.d.= not determined

Table 3.2: Physical and chemical characteristics of the soil in Makmur (0-150 cm)

Parameters	Soil depth (cm)				
	0-10	10-30	30-50	50-100	100-150
Bulk density (g cm^{-3})[1]	1.1 (0.0)	1.2 (0.0)	1.3 (0.1)	1.3 (0.0)	1.3 (0.0)
Sand (%)[2]	58.3 (0.7)	57.3 (0.6)	61.7 (1.4)	71.0 (2.7)	69.4 (0.9)
Silt (%)[2]	33.4 (1.7)	34.5 (2.2)	31.1 (2.3)	25.5 (4.1)	25.8 (1.8)
Clay (%)[2]	8.3 (2.3)	8.2 (2.8)	7.2 (3.2)	3.5 (1.8)	4.8 (2.1)
pH (H_2O)	5.8 (0.3)	5.9 (0.0)	5.3 (0.1)	5.3 (0.0)	5.4 (0.0)
pH (KCl 0.5*M*)	4.3 (0.1)	4.1 (0.1)	4.1 (0.1)	4.1 (0.1)	4.1 (0.1)
C (g 100 g^{-1})[3]	1.5 (0.1)	1.1 (0.1)	0.8 (0.1)	0.5 (0.1)	0.2 (0.0)
N (g 100 g^{-1})[3]	0.12 (0.01)	0.08 (0.01)	0.05 (0.01)	0.04 (0.01)	0.02 (0.00)
C/N	12.6 (0.6)	14.5 (0.9)	15.6 (1.1)	13.6 (1.3)	9.7 (0.9)
Available P (mg kg^{-1})[4]	3.8 (0.5)	2.2 (0.7)	1.0 (0.2)	1.1 (0.2)	1.4 (0.02)
K^+ ($cmol_c$ kg^{-1})[5]	0.08 (0.02)	0.02 (0.01)	n.d.	n.d.	n.d.
Na^+ ($cmol_c$ kg^{-1})[5]	0.03 (0.01)	0.03 (0.00)	0.09 (0.05)	0.06 (0.01)	0.08 (0.2)
Ca^{++} ($cmol_c$ kg^{-1})[5]	4.3 (0.5)	3.2 (0.7)	2.6 (0.5)	2.3 (0.6)	2.8 (0.6)
Mg^{++} ($cmol_c$ kg^{-1})[5]	1.0 (0.2)	0.5 (0.2)	0.3 (0.2)	0.4 (0.2)	0.7 (0.2)
Al^{+++} ($cmol_c$ kg^{-1})[6]	13.8 (1.0)	14.6 (0.1)	13.8 (0.7)	10.8 (1.4)	10.1 (1.1)
ECEC ($cmol_c$ kg^{-1})[5]	19.2 (0.5)	18.3 (1.0)	16.8 (1.3)	13.6 (1.9)	13.6 (1.8)
Base saturation (%)[7]	28.2 (3.9)	19.7 (4.1)	17.3 (2.6)	19.7 (3.7)	25.6 (2.6)
Fe (mg kg^{-1})[8]	135 (1.4)	143 (1.4)	n.d.	n.d.	n.d.
Zn (mg kg^{-1})[8]	4.5 (0.5)	3.5 (0.4)	n.d.	n.d.	n.d.
Mn (mg kg^{-1})[8]	70.3 (2.0)	57.8 (2.8)	n.d.	n.d.	n.d.

Soil analysis methods: 1) gravimetric; 2) pipette; 3) dry combustion; 4) CAL-method; 5) Mehlich; 6) calculated; 7) calculated; 8) NH_4OAc; Value in parentheses represents standard error of the means; n.d.= not determined

At both sites, the values of most soil chemical discriptions declined significantly with depth, except for exchangeable Al^{+++} in Kaduwaa and exchangeable Na^+ and base saturation in Makmur. With regard to soil organic C, mineral N, and base saturation, the soil in Kaduwaa can be considered more fertile than that in Makmur. This may be related to the shorter period of time the Kaduwaa site was under cultivation. The concentration of Fe and Mn were higher in Makmur than in Kaduwaa.

Pearson's correlation coefficients between soil parameters (pooled data of both sites, Table 3.3) show that the clay content was positively correlated with N, C, and available P. The N was positively correlated with pH (KCl 0.5*M*), available P and effective cation exchange capacity (ECEC). The C content was highly correlated with N, available P and ECEC, which in turn showed a positive correlation with pH (KCl 0.5*M*), but a negative one with bulk density. These relationships underline the limited role of clay minerals and the relative importance of soil organic matter in nutrient storage and retention and thus the determination of soil fertility. It can be seen that soil

N, available P and ECEC are generally more strongly correlated to the soil carbon than to the clay fraction.

Table 3.3: Pearson's correlation coefficients between soil parameters (pooled data of both sites, 0-150 cm)

X	Y	R^2	*P*
Clay (%)	N (g 100 g^{-1})	0.55	0.002
Clay (%)	C (g 100 g^{-1})	0.50	0.005
Clay (%)	Available P (mg kg^{-1})	0.72	0.001
C (g 100 g^{-1})	Available P (mg kg^{-1})	0.80	0.001
C (g 100 g^{-1})	ECEC ($cmol_c$ kg^{-1})	0.85	0.001
C (g 100 g^{-1})	N (g 100 g^{-1})	0.97	0.001
N (g 100 g^{-1})	pH (KCl 0.5*M*)	0.73	0.001
N (g 100 g^{-1})	Available P (mg kg^{-1})	0.82	0.001
N (g 100 g^{-1})	ECEC ($cmol_c$ kg^{-1})	0.85	0.001
ECEC ($cmol_c$ kg^{-1})	BD (%)	-0.63	0.001
ECEC ($cmol_c$ kg^{-1})	pH (KCl 0.5*M*)	0.59	0.001
ECEC ($cmol_c$ kg^{-1})	Available P (mg kg^{-1})	0.67	0.001

ECEC = Effective cation exchange capacity; BD=bulk density; pH= soil acidity

3.1.3 Climate

During the experimental period (July 2002 – June 2003) the precipitation was 1645 and 1317 mm yr^{-1} in Kaduwaa and Makmur, respectively (Figure 3.4). A relatively dry period occurred from July-October 2002 and from January-February 2003 (monthly precipitation <100 mm). Wet months occurred from November-December 2002 and from March-June 2003 (monthly precipitation >100 mm). The mean temperature during the experimental period was 21.2 ^oC in Kaduwaa and 23.8 ^oC in Makmur. The lower mean temperature in Kaduawaa than in Makmur was mainly due to the fact that the Kaduwaa site is at a higher altitude (1100 m asl) than the Makmur site (550 m asl).

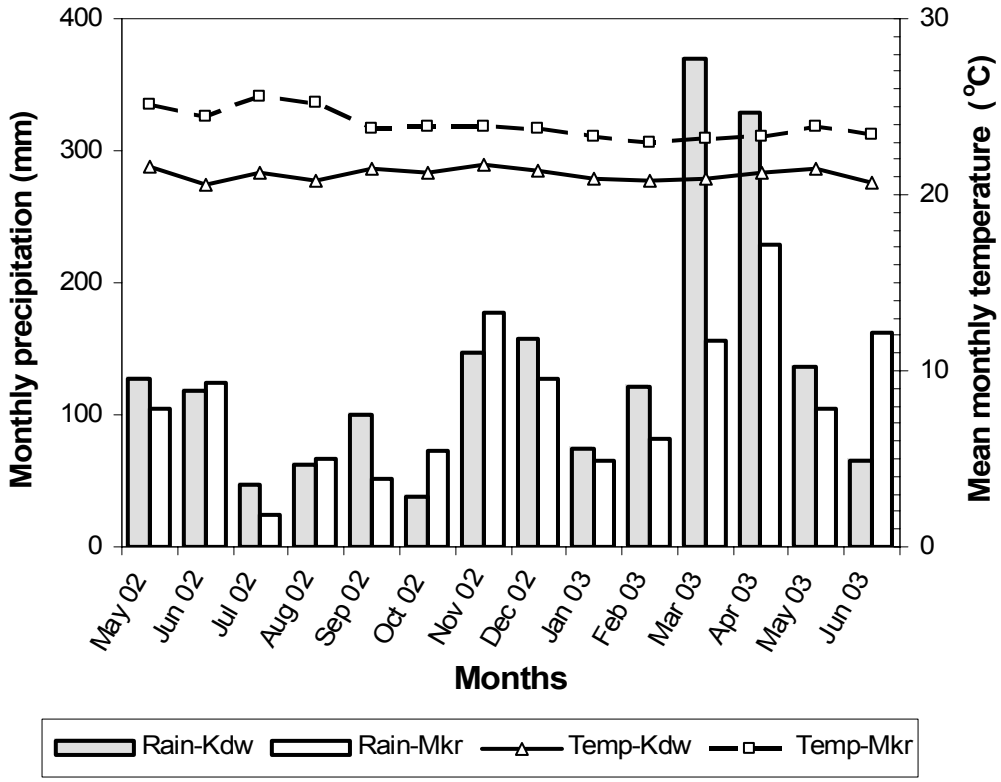

Figure 3.4: Monthly precipitation and temperature during study period in Kaduwaa and Makmur; Kdw = Kaduwaa and Mkr = Makmur (Source: STORMA meteorological data (Station Wuasa for Kaduwaa) and IMPENSO meteorological data (Station Bariri for Makmur))

3.2 Experimental design

Experiments were carried out in a randomized complete block design with five replicates. The research was threefold: (1) plot-wise enrichment with ^{15}N-Ammonium-^{15}N-Nitrate, (2) plot-wise ^{15}N natural abundance, and (3) glasshouse studies for the determination of plant-available ^{15}N with depth, the determination of the ^{15}N abundance of the 100%-fixing legumes (*B*-value) and the studies of the infection potential of the soils.

3.2.1 Nitrogen-15 enrichment method

Plot layout

Due to the limited choice of reference plants in the cacao agroforestry system, cacao and coffee were chosen as reference plants in Kaduwaa, and cacao, vanilla (*Vanilla planifolia*) and the perennial shrub-weed sida (*Sida retusa L.)* were used as reference

plants in Makmur. The soil of an area of 3 m x 6 m in Kaduwaa and 4 m x 6 m in Makmur around each of these plants was labeled with ^{15}N (Figure 3.5).

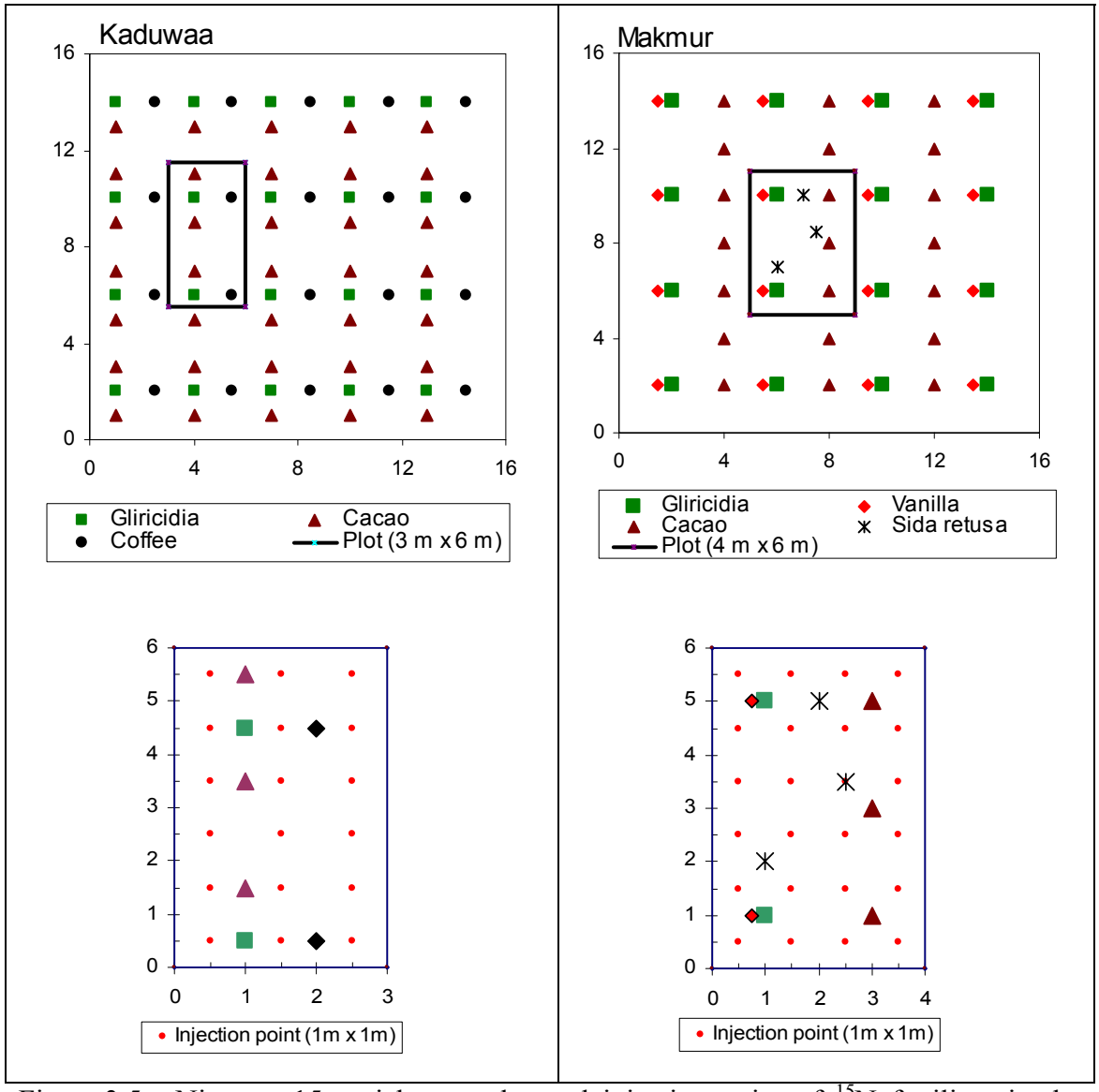

Figure 3.5: Nitrogen-15 enrichment plot and injection point of ^{15}N fertilizer in the cacao agroforestry systems in Kaduwaa and Makmur

At each site, five plots were labeled with 10 kg N ha^{-1} of ^{15}N-ammonium-^{15}N-nitrate fertilizer containing 10-atom-% ^{15}N. The enrichment of ^{15}N was 0.106 g m^{-2} (or 2.84 g m^{-2} ^{15}N-ammonium-^{15}N-nitrate). In the enriched plots, the plant roots were isolated from the surrounding soil by excavating the plot boundaries to a depth of 1 m and inserting a thick tarpaulin. It was assumed that this would prevent lateral migration

of ^{15}N fertilizer while also hindering lateral root growth beyond the enriched zone and the penetrating of roots from the unlabelled zone.

Labeling ^{15}N enrichment

To ensure homogeneous distribution of the ^{15}N enriched fertilizer, the following strategy for its application was chosen: First of all, the application was split: the first half was applied at the beginning of the study and the second half at six months later. The exact proportion of ^{15}N labeled fertilizer was matched with the actual vertical soil N_{tot} gradient found in the soils. From the data of the basic soil analyses (Table 3.1 and 3.2), the proportion of soil N_{tot} gradient at each depth was 40.9, 38.6, 9.6, and 10.9 % for the soil depths of 0-10, 10-30, 30-50, and 50-100 cm, respectively. Therefore, for each application, 0.58, 0.55, 0.14 and 0.15 g of ^{15}N-ammonium-^{15}N-nitrate was dissolved in 50 ml water and then applied to the corresponding soil depth (m^{-2}). Details are shown in Table 3.4.

Table 3.4: Concentration of ^{15}N-ammonium-^{15}N-nitrate for every depth at each time of application/injection

Soil depth (cm)	N_{tot} (%)	Rate of ^{15}N-ammonium-^{15}N-nitrate (g m^{-2})
0-10	40.9	0.58
10-30	38.6	0.55
30-50	9.6	0.14
50-100	10.9	0.15
Total	100.0	1.42

The labeled fertilizer was injected into the soil with a special injection device (Figure 3.6), consisting of a 1-m steel tube of 1.5 cm in diameter, with 12 tin nozzles at the top side of the tube. Aliquots of dissolved ^{15}N-labeled fertilizer were injected under pressure (3 bars) successively at 5, 20, 40 and 75 cm soil depth at horizontal distances of 1 m by 1 m (rectangular grid). For injection into the top 0-10 cm, only 6 tin nozzles were used, while the remaining nozzles were closed with plastic tape to avoid squirting above the soil. It was assumed that lateral diffusion of the applied ^{15}N fertilizer was sufficiently homogenous for labeling of the total plot.

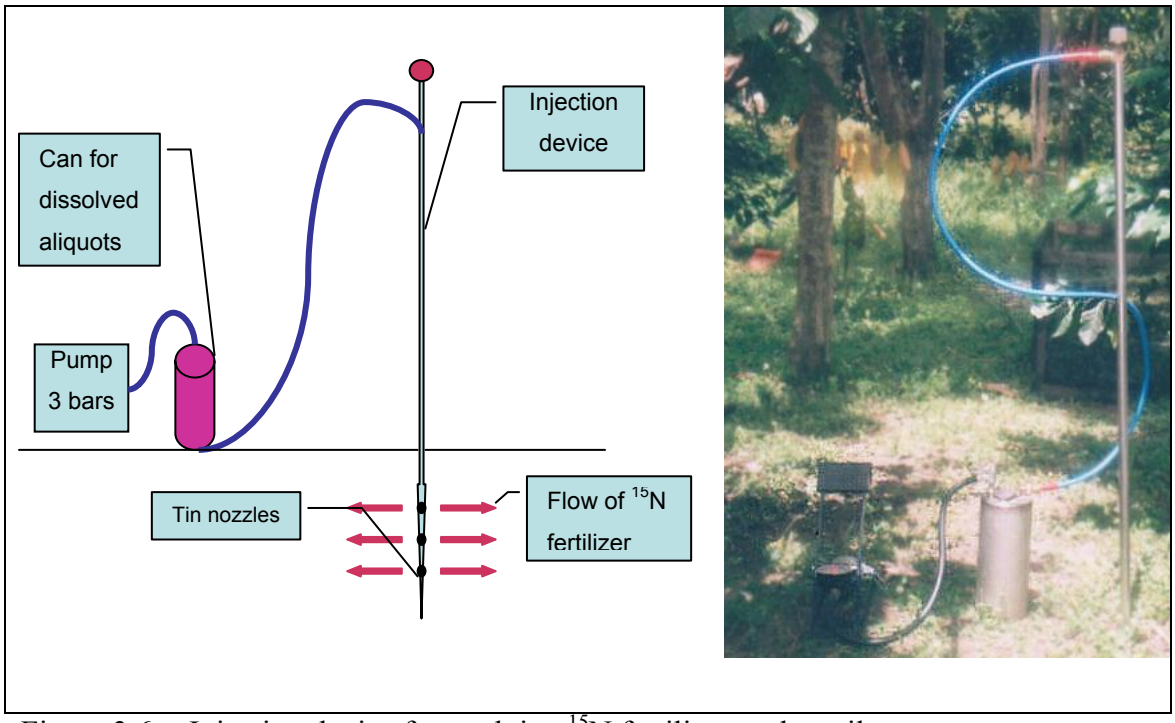

Figure 3.6: Injection device for applying ^{15}N fertilizer to the soil

Data processing and synthesis

The share of N in the biomass that was derived from the process of BNF as expressed by McAuliffe et al. (1958) and Chalk (1985) is given in equation 1:

$$\%\ \text{Ndfa} = \left(1 - \frac{\text{Atom\% } ^{15}\text{N excess legume}}{\text{Atom\% } ^{15}\text{N excess reference plant}}\right) \text{x} \quad 100 \qquad [1]$$

where %Ndfa is the percentage of N derived from atmospheric N_2, and atom% ^{15}N excess is the value of the samples after subtracting %^{15}N atmosphere (standard, 0.3663).

3.2.2 Nitrogen-15 natural abundance method

At the same site, the ^{15}NNAM was used on five parallel replications. Treatments were the same as described for the ^{15}NEM except that in the ^{15}NNAM plots no ^{15}N fertilizer was applied.

Data processing and synthesis

The isotope values are generally expressed in the delta notation ($\delta^{15}N$), defined as the one per thousand deviations from the ^{15}N abundance of atmospheric N_2 (equation 2) (Shearer and Kohl 1986).

$$\delta^{15}N[‰] = \frac{\text{atom}\%\ ^{15}N_{(\text{sample})} - \text{atom}\%\ ^{15}N_{(\text{Standard})}}{\text{atom}\%\ ^{15}N_{(\text{Standard})}} \cdot 1000 \quad [2]$$

where atom% ^{15}N standard is the enrichment of atmospheric N_2, which is constant at an abundance of 0.3663 atom% ^{15}N (Mariotti et al. 1983). Positive $\delta^{15}N$ values denote ^{15}N-enrichment relative to the standard, while negative $\delta^{15}N$ values denote ^{15}N-depletion relative to the standard. Hence by definition, the $\delta^{15}N$ value of air is zero. The %Ndfa is calculated according to equation 3 (Shearer and Kohl 1986):

$$\%\,\text{Ndfa} = \frac{\delta^{15}N_{\text{reference}} - \delta^{15}N_{\text{legume}}}{\delta^{15}N_{\text{reference}} - \delta^{15}N_{B\text{-value}}} \cdot 100 \quad [3]$$

where $\delta^{15}N$ reference is the $\delta^{15}N$ value of the reference plant, $\delta^{15}N$ legume is the $\delta^{15}N$ value of the fixing plant and *B*-value is the $\delta^{15}N$ value of the fixing plant grown with N_2 as the sole N source.

Plant-available soil ^{15}N with depth

In order to determine the natural enrichment of plant-available soil ^{15}N with depth, soil at depths of 0-10, 10-30, 30-50, 50-100 and 100-150 cm was sampled from each plot with an auger. One mixed sample of the soil was air-dried at room temperature for 3-4 days and then sieved to 2 mm to remove roots and stones. The soil (around 2 kg) was put in plastic pots and then watered with $H_2O_{dest.}$ to field capacity. Eight seeds of *Oryza sativa* L. variety 'gogo' were planted in per pot. After one week, the paddy was thinned to 4 plants per pot, which were and left to grow for 6 weeks. At this stage, the plants were uprooted and washed using $H_2O_{dest.}$ to remove the soil from the roots. Samples were dried at 50 °C for 72 h, until constant weight. Plants were finely ground in a piston

ball mill and analyzed for $\%^{15}N$ using an ANCA mass spectrometer (SL 20-20, PDZ Europa).

Nitrogen-15 discrimination (*B*-value)

The *B*-value of Gliricidia was determined in trees grown in the glasshouse without soil-derived N-nutrition and fully dependent on atmospheric N_2. To establish a N-free medium, sand from a nearby riverbank was used, watered with running water for 5 minutes, and placed into 10-liter containers with 5 l water. The sand was washed and stirred by hand and sieved twice until clean. Finally, the sand was washed (twice) with $H_2O_{dest.}$, air-dried and filled into the plant containers containing 5 kg sand per pot.

Four Gliricidia seeds were planted per pot. They were thinned to two plants per pot two weeks after planting. The pots were irrigated daily with 200 ml $H_2O_{dest.}$ and fertilized with 100 ml Hoagland N-free nutrition solution (Table 3.5) once a week until 4 weeks, then every 3 days for the rest of the treatment. The Hoagland solution was prepared according to Gibson (1980) and Thielen-Klinge (1997).

Table 3.5: Hoagland N-free nutrition solution

Hoagland solution	Amount ml		Concentration (g/l)
$H_2O_{dest.}$	(in 1000)		
K_2HPO_4	1.1	1M	0.19
KCl	3.9	1M	0.29
$CaCl_2.2H_2O$	5.0	1M	0.73
$MgSO_4.7H_2O$	1.5	1M	0.37
Micronutrient[$]	1.0		
Fe EDTA[§]	1.0		

[$]Micronutrient was prepared as shown in Table 3.6. [§]The FeEDTA (Fe-ethylenediaminetetraacetic acid) solution was prepared from 17.2 g of $FeSO_4.7H_2O$ mixed with 250 ml 1 N KOH (5 minutes under lighter mixing). Then 22.8 g of EDTA was added in 1000 ml of water and aerated vigorously overnight.

Table 3.6: Micronutrients of in Hoagland N-free nutrien solution

Micronutrient	Concentration (g/l)
$H_2O_{dest.}$	(in 1000)
$ZnSO_4.7H_2O$	0.22
$CuSO_4.5H_2O$	0.08
$MnCl_2.4H_2O$	1.81
H_3BO_3	2.86
$Na_2MoO_4.4H_2O$	0.02

Plants were inoculated with a soil suspension from the two sites, i.e., a mixture of topsoil from Kaduwaa and a mixture of topsoil from Makmur. The inoculant was prepared by adding 250 ml of $H_2O_{dest.}$ to 5 g of the topsoil mixture from each site, and shaken for 30 minutes. It was left to settle before being applied at a rate of 10 ml pot^{-1} two and four weeks after germination. At 12, 24, and 36 weeks after planting, five pots for each treatment were sampled, the plants were uprooted, washed in $H_2O_{dest.}$ to remove adhering soil and separated into leaves, twigs/stems, nodules and roots, and weighed. Litter from falling leaves was also collected throughout the experiment. Samples were dried at 50 °C for 72 h, or until constant weight. Plants were finely ground in a piston ball mill and analyzed for %N and $\%^{15}N$ using an ANCA mass spectrometer (SL 20-20, PDZ Europa).

3.2.3 Infection potential

To study the infection potential of the soils ~3 kg (mixture of soil randomly collected) of the upper 20 cm (~A-horizon) was collected at both sites either in the direct vicinity of, or at least 4 m from a NFT. The infection potential was assessed using mungbean (*Vigna radiata*) and cowpea (*Vigna unguiculata*) as trap plant for Gliricidia rhizobia. Four seeds pot^{-1} of each indicator legume were seeded. After one week, two plants were selected and grown until the flowering stage. Then the plants were uprooted and washed with tap water to remove adhering soil. The nodules from each plant were removed and the fresh weight and nodule number was determined.

3.2.4 Aboveground biomass

Destructive and non-destructive determination of aboveground biomass of Gliricidia was carried out. In order to develop an allometric equation of aboveground biomass of Gliricidia, three trees at each site and two trees from Biromaru (surrounding site at 100 m asl, to increase the number of trees for the equation) were selected. The basal diameter (D) of the trees and their height were measured. Each tree was separated into four fractions: (1) leaflets, (2) twigs (D < 2 cm), (3) small branches (2 cm < D < 5 cm), (4) large branches and stems (D > 5 cm). These fractions were chosen based on the observation that tree components showed varying nutrient concentrations and the basal diameter of the trees was mostly > 5 cm.

Since the farmers did not permit cutting at the base of Gliricidia trees, two different methods for determining the total aboveground biomass were applied. First, for the base of the trees, the diameter and height until the first branching point was measured to calculate the volume, and the result was multiplied with the density of the fraction $D > 5$ cm. Second, the rest of the trees were separated into the above-mentioned fractions and fresh weights recorded. For the determination of the moisture content of each fraction, sub-samples were taken.

The following model was used for deriving Gliricidia biomass (dry matter) and N content from the basal diameter, with a and b fitting numerically using Sigma Plot™ software.

$$\text{Ln (Individual tree biomass [kg])} = a + b\ \text{Ln}(D) \quad [5]$$

$$\text{Ln (Individual tree N [g])} = a + b\ \text{Ln}(D) \quad [6]$$

Five plots of 12 m x 12 m on each site were marked and all diameters of the plants recorded and the plants separated into species. The (annual) input of N via litter of the leguminous trees was determined, based on the N content of the litter collected in litter traps (1 m x 1 m size with 5 replicates). Additionally, the biomass and N content of Gliricidia pruning was determined.

3.3 Sampling methods and analyses

3.3.1 Plant samples

Samples of young leaves (leaflet number two and three from the tip of fully developed leaves), wooden compartments (twigs) and litter of fixing and reference plants were collected initially at both sites and in all plots. Leaf and litter samples[1] from the enriched and natural abundance plots were taken at 12-week intervals (4 samplings). Samples were placed in paper bags and oven-dried at 50 °C for 72 h or until constant weight, then finely ground to pass through a 1 mm sieve and analyzed for %N and $\%^{15}N$ using an ANCA mass spectrometer (SL 20-20, PDZ Europa).

[1] Woody compartments are not supposed to vary considerably during the observation period and, therefore, were only sampled twice, i.e., at the beginning of the experiment and 12 weeks later.

All samples of the ^{15}NEM and ^{15}NNAM were treated separately and handled with care to avoid contamination between treatments. The preparation was started with those plant components that were expected to have the lowest ^{15}N contents. All equipment was washed with water, rinsed once with Aceton, then rinsed twice with $H_2O_{dest.}$, and dried before moving to the preparation of the next sample.

3.3.2 Soil samples

Soil ^{15}N, N_{tot} and basic soil characteristics

The ^{15}N content of total soil N at 0-10, 10-30, 30-50, 50-100 and 100-150 cm depths was determined initially in the ^{15}NNAM plots at both sites and repeated at 12-week intervals (altogether 5 samplings). In the ^{15}NEM plots, these measurements started 12 weeks after the enrichment of ^{15}N fertilizer and were repeated in 12-week intervals (4 samplings). Soil was sampled using a soil auger (Eijkelkamp) from five points depth^{-1} and plot^{-1}. An air-dried, stone-free mixture of the soil was finely ground (0.2 mm) using a piston ball mill (Rechsch PM-4000) and analyzed for %N and %^{15}N using an ANCA mass spectrometer (SL 20-20, PDZ Europa).

Soil mineral ^{15}N and N

Soil mineral ^{15}N ($^{15}N_{min}$) and N (N_{min}) at the ^{15}NEM and ^{15}NNAM plots was analyzed at the end of experiment (Jun-03) to determine the persistence/fade of soil labeling within the main rooting zone (0-1.5 m). Twenty-five grams of soil (depths as above) was extracted with 100 ml of 0.5 *M* K_2SO_4 by shaking the samples for 1 hour and filtering the extract through pre-washed (0.5 *M* K_2SO_4) filter papers. The concentration of NH_4^+ and NO_3^- in the extract was analyzed using continuous flow injection colorimetry (Cenco instruments, Breda, Netherland). Soil moisture was determined gravimetrically. Nitrogen-15 in the extract was analyzed by the diffusion method as described in detail by Stark and Hart (1996). However, instead of letting the acid traps float on the solution surface, two pieces of 5-cm diameter teflons were used to encase the acidified filter discs (2 discs of 7 mm diameter cut from glass-fiber filter paper and acidified with 20 µl of 2.5 *M* K_2SO_4). The $^{15}NH_4^+$ in the samples was determined by filling 50 ml of the soil extract (containing at least 20 µg NH_4^+) into 150 ml glass bottle. MgO was added to convert NH_4^+ to NH_3 and then acid trap was immediately put between the teflon on the

top of the bottle and the lid fastened. The solution was shaken twice a day for 6 days. The $^{15}NO_3^-$ in the samples was determined after having kept the bottles (used for $^{15}NH_4^+$) opened for 3 days to get rid of the NH_4^+. Devarda's alloy was added to convert NO_3^- to NH_3 and the acid trap was immediately put between the teflon on the top of the bottle and the lid fastened. The solution was shaken twice a day for 6 days. The acid trap was placed in the tin capsules for $^{15}NH_4^+$ and $^{15}NO_3^-$. The $^{15}NH_4^+$ and $^{15}NO_3^-$ was analyzed using EA-IRMS (Finigan MAT, Bremen, Germany).

3.4 Statistical analysis

Two factorial analyses of variance (general linear model [GLM] using SPSS-11) of the randomized complete block design (RCBD) were used to compute differences in atom% ^{15}N excess, $\delta^{15}N$ values, $\%N_{tot}$, N_{min} and $^{15}N_{min}$ in soil and plants and %Ndfa of both methods. A GLM was also applied for the randomized complete design (RCD) to compute differences in $\delta^{15}N$ values and $\%N_{tot}$, and *B*-values of Gliricidia under greenhouse study; nodule fresh weights, nodule numbers and infection potentials. The paired *t*-Test was used to compare the %Ndfa of both methods. Prior to analyses, all data were tested for normality by the Kolmogorov-Smirnov *Z*-Test. Data of $\%N_{tot}$ and $\delta^{15}N$ value of the soil and $\delta^{15}N$ of the *B*-value were not normally distributed, and hence a square root transformation was performed for percentage data; all other data sets were $\log_{10}$-transformed (Gomez and Gomez 1984). All data were reported on a re-transformed scale for comparison of treatment means. When the treatment means were statistically different, the LSD comparison of means was used.

Table 3.7: Statistical analysis of data

Data	Transformation	Statistics	Independent variables
Soil physical and chemical characteristics	/	ANOVA (RCBD) LSD	Site and depth
		Pearson's correlations coefficient	Between soil parameter
Soil $\%N_{tot}$	square root	ANOVA (RCBD) LSD	Depth and time
Soil atom% ^{15}N excess	log10	ANOVA (RCBD) LSD	Depth, time and distance
Soil $\delta^{15}N$	log10	ANOVA (RCBD) LSD	Depth and time
Soil N_{min} Soil $^{15}N_{min}$	/ square root	ANOVA (RCBD) LSD	Site and depth
Plant $\%N_{tot}$	/	ANOVA (RCBD) LSD	Species and time (every plant part)
Plant atom% ^{15}N excess	/	ANOVA (RCBD) LSD	Species and time (every plant part)
Plant $\delta^{15}N$	/	ANOVA (RCBD) LSD	Species and time (every plant part)
B-value ($\delta^{15}N$)	log10	ANOVA (RCD) LSD	Soil solution and Plant part
%Ndfa	/	ANOVA (RCBD) LSD *t*-Test	Plant (reference) and time ^{15}NNAM vs ^{15}NEM
Infection potential	/	ANOVA (RCD) LSD	Soil and crop
Total dry matter (litter)	log10	ANOVA (RCBD)	Site and time
Total N accumulation (litter)	square root	ANOVA (RCBD)	Site and time
Dry matter, total N and basal diameter	ln-transformed	Linear regression	

4 RESULTS AND DISCUSSION

4.1 Nitrogen-15 enrichment method

4.1.1 Atom% ^{15}N excess and $\%N_{tot}$ in soil

Vertical and temporal variations of atom% ^{15}N excess

The atom% ^{15}N excess in the soil at both sites, Kaduwaa and Makmur, differed significantly ($P<0.01$) with soil depth and time of sampling. The $\%N_{tot}$ was only affected by soil depth ($P<0.01$). There was a significant interaction between soil depth and time of sampling on atom% ^{15}N excess in the soil at both sites ($P<0.01$) and on $\%N_{tot}$ in the soil in Kaduwaa ($P<0.01$; Appendix 2).

In Kaduwaa, the highest atom% ^{15}N excess in the soil over time was in the top soil layer (0-30 cm), which was significantly higher ($P<0.01$) than in the lower layer (30-150 cm). A similar trend was also found in Makmur; the atom% ^{15}N excess in the top soil (0-10 cm) was significantly higher ($P<0.01$) than the lower layer (10-150 cm) (Figure 4.1). This was caused by the ^{15}N enrichment, which was based on the $\%N_{tot}$ in the soil. Nearly 40 % of the ^{15}N fertilizer was injected into the top 10 cm and 10-30 cm layer, and only 10 % into the 30-50 cm and 50-100 cm layers, respectively (Table 3.4). Consequently, decreasing atom% ^{15}N excess with depth was correlated with decreasing $\%N_{tot}$ with depth. The $\%N_{tot}$ in the soil of the enrichment plots at both sites declined substantially with depth from 0.21 and 0.15 % in the top soil in Kaduwaa and Makmur, respectively, to 0.3 % in the 50-150 cm soil depth (Figure 4.1).

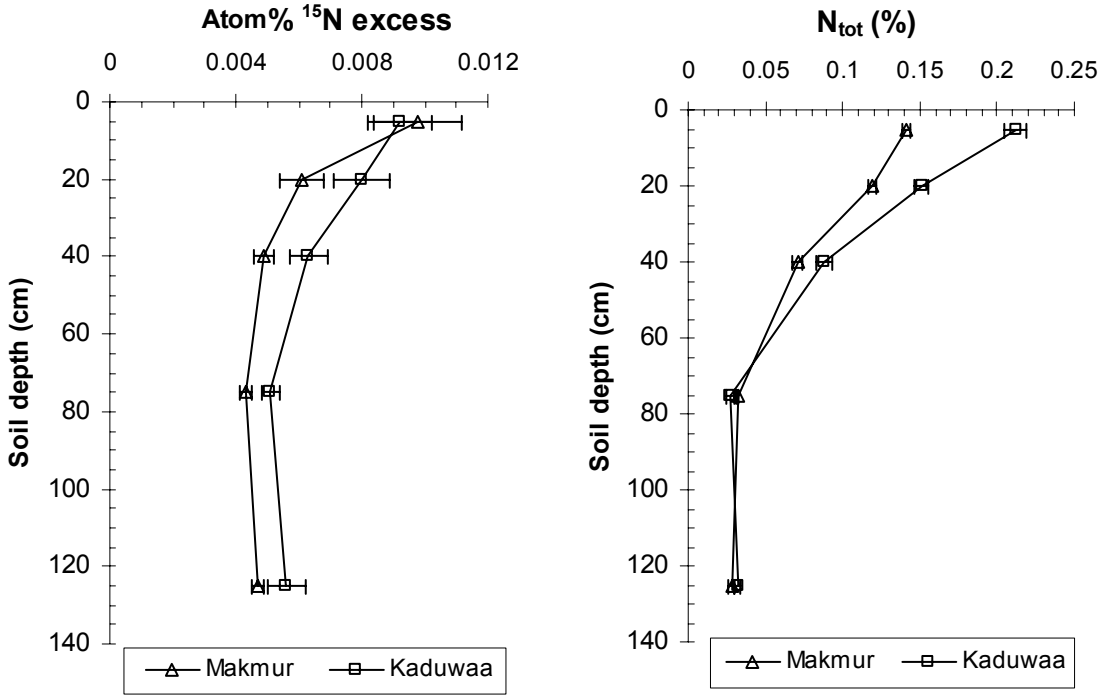

Figure 4.1: Mean atom% ^{15}N excess and $\%N_{tot}$ in the soil at different depths in Kaduwaa and Makmur (n=20, bars represent standard error of the means)

Detailed trends of changing atom% ^{15}N excess in the soil at both sites at different times of sampling are presented in Figure 4.2. The enrichment of 10-atom% $^{15}NH_4^+$-$^{15}NO_3^-$ at 5 kg ha^{-1} (Jul-02 and Dec1-02) increased significantly ($P<0.01$) the atom% ^{15}N excess in the soil in the 0-30 cm depth in contrast to the 30-100 cm depth. Four weeks after the enrichment with ^{15}N fertilizer (Aug-02 and Jan-03), the atom% ^{15}N excess in the soil in a depth of 0-30 cm declined rapidly to the same value as and even lower than in the 30-100 cm depth, and the atom% ^{15}N excess in the whole soil profile (0-100 cm) was not significantly different (Appendix 3). Twelve and 24 weeks after the enrichment with ^{15}N fertilizer (Oct-02, Dec-02 and Mar-03, Jun-03, respectively), the atom% ^{15}N excess of the soil returned to the level of natural abundance, but still differed significantly ($P<0.01$) with depth.

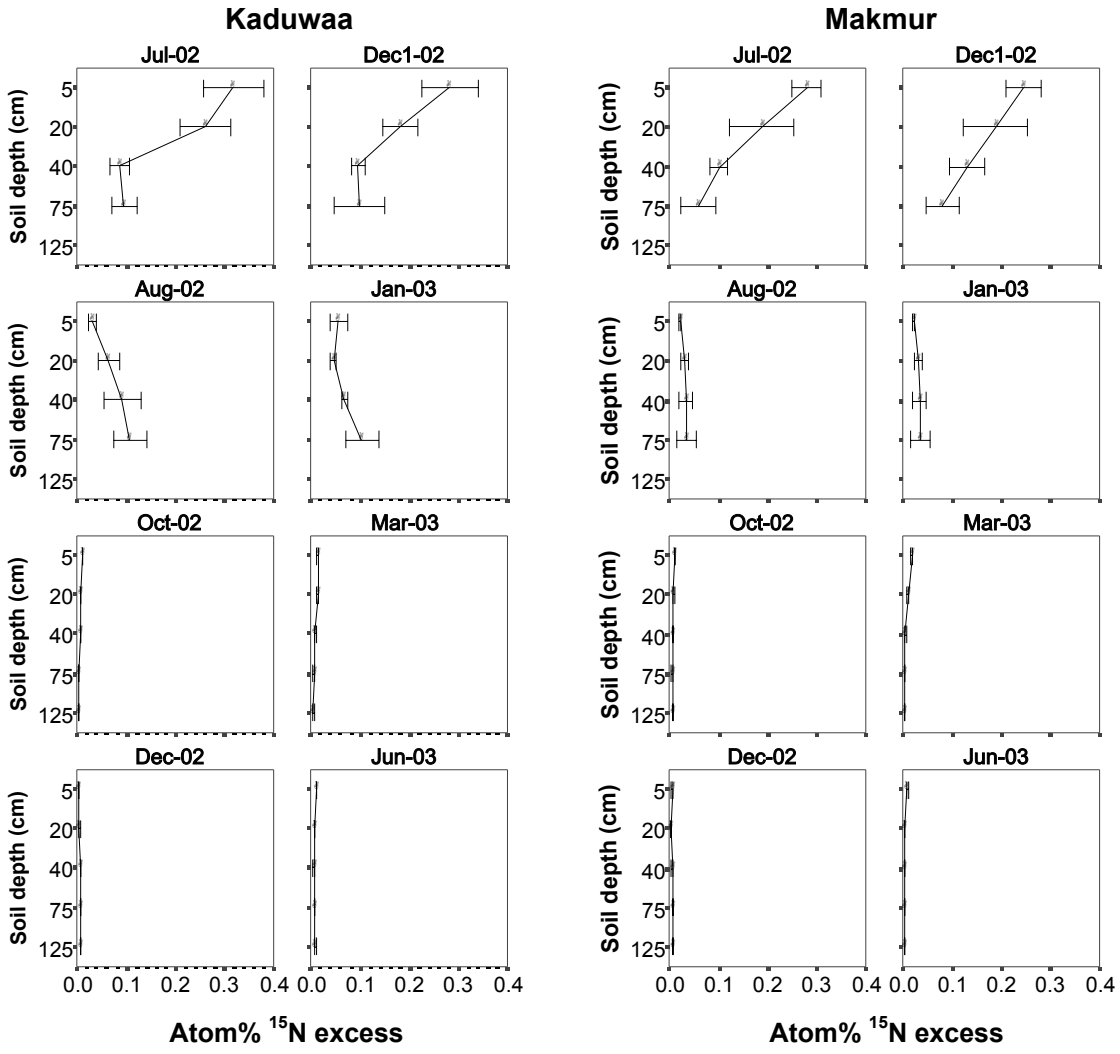

Figure 4.2: Changes in atom% ^{15}N excess in soil at different depths and times of sampling after enrichment with $^{15}NH_4^+$-$^{15}NO_3^-$ in Kaduwaa and Makmur (n=3 for data Jul-02, Aug-02, Dec1-02 and Jan-02; n=5 for data Oct-02, Dec-02, Mar-03 and Jun-03; bars represent standard error of the means)

The strategy of splitting the enrichment with ^{15}N fertilizer into two applications increases the availability of labeled ^{15}N to the plant. Some researchers also use multiple applications of ^{15}N fertilizer to maintain reasonably consistent ^{15}N available from ^{15}N fertilizer to fixing and reference plants over prolonged periods, e.g., in pastures (Steele and Littler 1987) and tree legumes (Peoples et al. 1996).

Lateral variations of the atom% ^{15}N excess

Equal distribution of ^{15}N fertilizer laterally and vertically is crucial in ^{15}NEM. In order to trace the movement of ^{15}N fertilizer laterally and vertically, soil samples were taken 3 hours after the enrichment (0 day) and 28 days later (28 days) for both the first and the second application, and at distance 0, 15 and 30 cm from the point of ^{15}N fertilizer injection. Analysis of variance (ANOVA) of the effect of distance from the injection point on atom% ^{15}N excess in the soil in Kaduwaa at 0 and 28 days after the injection is presented in Appendix 4. The results are presented in Figure 4.3.

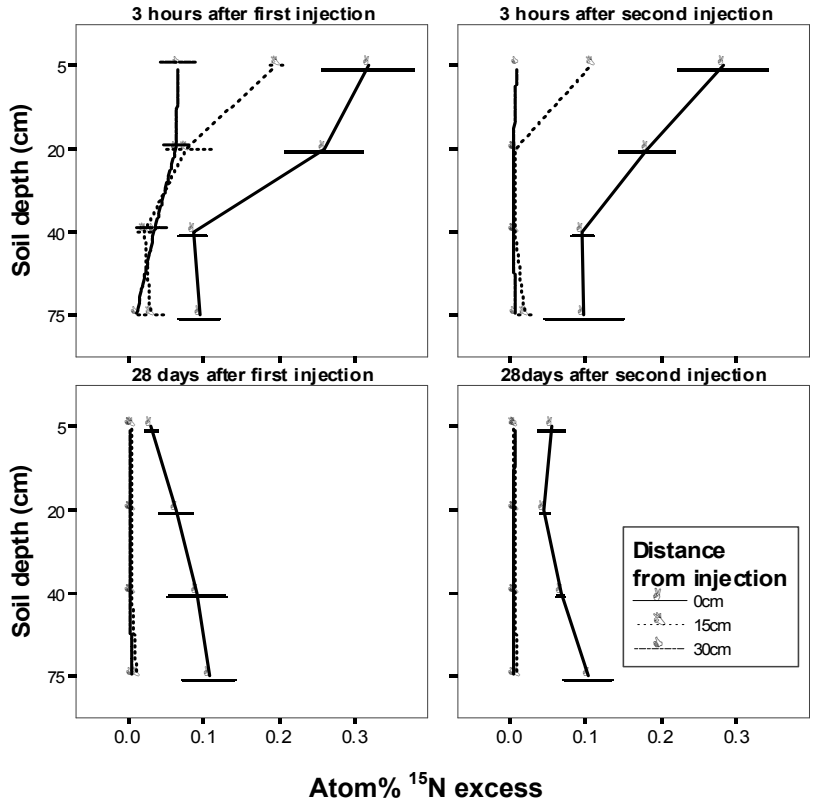

Figure 4.3: Changes in atom% ^{15}N excess in soil from point of injection of $^{15}NH_4^+$-$^{15}NO_3^-$ in Kaduwaa at different depths (n=3, bars represent standard error of the means)

The injection of ^{15}N fertilizer was not as successful as expected. There was little lateral movement of ^{15}N fertilizer. In contrast to that at the point of injection (0 cm), the atom% ^{15}N excess in the soil at 15 and 30 cm from the point of fertilizer injection had not increased (still at the level of natural abundance) even at 28 days after the enrichment. Most changes in ^{15}N fertilizer occurred at the 0-30 cm depth. At both depths, the atom% ^{15}N excess declined rapidly to levels below that of the atom% ^{15}N

excess in deeper soil layers. This decline may have been caused by plant uptake and ^{15}N fertilizer leaching to greater depths. At 28 days after the first and second enrichments, the atom% ^{15}N excess in the soil did not differ significantly with depth. In the study of injected ^{15}N fertilizer to different depths (15 and 100 cm depths) in mixed legume stands, Gathumbi et al. (2003) also observed that ^{15}N fertilizer was concentrated below the injection point with little lateral movement. Their results showed that soil samples taken 20 to 50 cm away from the ^{15}N injection point at five weeks after ^{15}N fertilizer application had ^{15}N enrichment similar to the background soil.

Vertical variations of mineral soil ^{15}N

The atom% ^{15}N excess of N_{min} in the soil of the enrichment plots at different depths was analyzed at the end of the experiment. The atom% $^{15}NH_4^+$ and $^{15}NO_3^-$ excess in the soil differed by site but were not affected by soil depth. The atom% $^{15}NH_4^+$ and $^{15}NO_3^-$ excess was higher in Kaduwaa than in Makmur (Table 4.1; ANOVA in Appendix 3). On the contrary, the NH_4^+ and NO_3^- concentrations in the soil decreased significantly with depth (Table 4.2; ANOVA in Appendix 5).

Table 4.1: Atom% ^{15}N excess of mineral N in enrichment plots in Kaduwaa and Makmur at different depths determined at end of experiment (Jun-03)

Soil depth	Kaduwaa		Makmur	
	$^{15}NH4^+$	$^{15}NO3^-$	$^{15}NH4^+$	$^{15}NO3^-$
(cm)	%	%	%	%
0-10	0.118 (0.086)	0.121 (0.084)	0.036 (0.004)	0.031 (0.008)
10-30	0.080 (0.030)	0.097 (0.034)	0.037 (0.012)	0.034 (0.009)
30-50	0.052 (0.020)	0.458 (0.017)	0.023 (0.009)	0.025 (0.014)
50-100	0.120 (0.053)	0.084 (0.049)	0.042 (0.021)	0.020 (0.008)
100-150	0.063 (0.026)	0.050 (0.007)	0.058 (0.038)	0.045 (0.025)
LSD (p= 0.05)	ns	ns	ns	ns

ns=not significant; n=3; values in parentheses represent standard error of the means

Table 4.2: Soil mineral N[§] in enrichment plot at different depths determined at end of experiment (Jun-03)

Soil depth (cm)	NH_4^+ mg kg^{-1}	NO_3^- mg kg^{-1}
0-10	11.0 (0.6) d	6.7 (1,3) b
10-30	7.4 (0.9) c	2.1 (0.3) a
30-50	4.8 (0.6) b	1.2 (0.1) a
50-100	1.4 (0.2) a	0.7[#]
100-150	1.9 (0.5) a	n.d.
LSD (p= 0.05)	1.4	2.2

[§]*Data from Kaduwaa and Makmur site were merged, since site did not affect soil N;* [#]*only detected in one replication; n.d. not detected; values within one column followed by the same letter are not significantly different at p < 0.05; n=6; values in parentheses represent standard error of the means*

4.1.2 Atom% ^{15}N excess and $\%N_{tot}$ in plants

Plant species and time of sampling significantly affected (P<0.01) the atom% ^{15}N excess and $\%N_{tot}$ in all plant parts at both sites, except for $\%N_{tot}$ in the litter in Kaduwaa. There was also a significant interaction of both factors on atom% ^{15}N excess and $\%N_{tot}$ in plants at both sites (Appendix 6 and 7).

Variations among plants and plant parts

In Kaduwaa, the mean atom% ^{15}N excess in the leaves of reference plants was significantly higher (P<0.01) than that of the fixing plant, with overall means and standard errors of 0.074±0.007, 0.191±0.016 and 0.206±0.026 % for Gliricidia, cacao and coffee, respectively. In Makmur, the values for the leaves of the reference plants were also significantly higher (P<0.01) than for Gliricidia, with overall means and standard errors of 0.051±0.006, 0.219±0.020, 0.145±0.023 and 0.108±0.021 % for Gliricidia, cacao, vanilla and sida, respectively (Table 4.3). The atom% ^{15}N excess in the twigs and litter at both sites showed similar trends as the values for the leaves. The atom% ^{15}N excess of the reference plants was 0.12-0.20 % higher in the twigs and 0.04-0.12 % higher in the litter compared than in the respective compartments of the fixing plant (P<0.01). Gliricidia showed the highest $\%N_{tot}$, irrespective of parts and sites (Table 4.4). Among the reference plants, the lowest $\%N_{tot}$ was found in cacao.

Table 4.3: Atom% ^{15}N excess in leaves, twigs and litter of fixing and reference plants in Kaduwaa and Makmur

Plant	Leaves	Twigs	Litter
		Atom% ^{15}N excess	
		Kaduwaa	
Gliricidia	0.074 (0.007) a	0.082 (0.165) a	0.051 (0.006) a
Cacao	0.191 (0.016) b	0.176 (0.015) b	0.093 (0.012) b
Coffee	0.198 (0.026) b	0.211 (0.031) b	0.150 (0.020) c
LSD (p=0.05)	0.022	0.062	0.022
		Makmur	
Gliricidia	0.073 (0.009) a	0.091 (0.021) a	0.079 (0.007) a
Cacao	0.191 (0.020) c	0.317 (0.081) b	0.212 (0.020) b
Vanilla	0.131 (0.019) b	nd	nd
Sida	0.114 (0.021) b	0.220 (0.038) b	nd
LSD (p=0.05)	0.032	0.108	0.016

Values within one column followed by the same letter are not significantly different at P < 0.05; n=20; nd = not determined; values in parentheses represent standard error of the means

Table 4.4: Proportion of N total (%N_{tot}) in leaves, twigs and litter of fixing and reference plants in in Kaduwaa and Makmur

Plant	Leaves	Twigs	Litter
		N_{tot} (%)	
		Kaduwaa	
Gliricidia	3.72 (0.14) c	1.70 (0.10) b	2.27 (0.03) b
Cacao	2.22 (0.09) a	1.02 (0.06) a	1.15 (0.06) a
Coffee	3.25 (0.09) b	1.67 (0.10) b	2.26 (0.05) b
LSD (p=0.05)	0.18	0.18	0.09
		Makmur	
Gliricidia	3.44 (0.11) d	1.47 (0.04) b	1.83 (0.06) a
Cacao	1.97 (0.08) b	0.79 (0.03) a	2.06 (0.10) b
Vanilla	1.05 (0.06) a	n.d.	n.d.
Sida	2.81 (0.15) c	1.50 (0.18) b	n.d.
LSD (p=0.05)	0.15	0.27	0.11

Values within one column followed by the same letter are not significantly different at P < 0.05; n= 20; n.d. = not determined; values in bracket represent standard error of the means

The atom% ^{15}N excess among reference plants also varied considerably depending on plant species and plant parts. In Kaduwaa, the atom% ^{15}N excess in the leaves and twigs generally did not differ between cacao and coffee. In Makmur, the values in the leaves of the reference plants differed significantly, with the highest atom% ^{15}N excess observed in cacao, while there were no differences in that in the twigs between cacao and sida. This may have been the result of different patterns of uptake of plant-available soil ^{15}N, resulting from the different rooting patterns of the

reference plants. Cacao and coffee root deeper than vanilla and sida and are thus able to absorb more soil ^{15}N from deeper layers.

Variations at different plant parts and times of sampling

At both sites, the atom% ^{15}N excess in the leaves of fixing and reference plants changed with the sampling date (Figure 4.4).

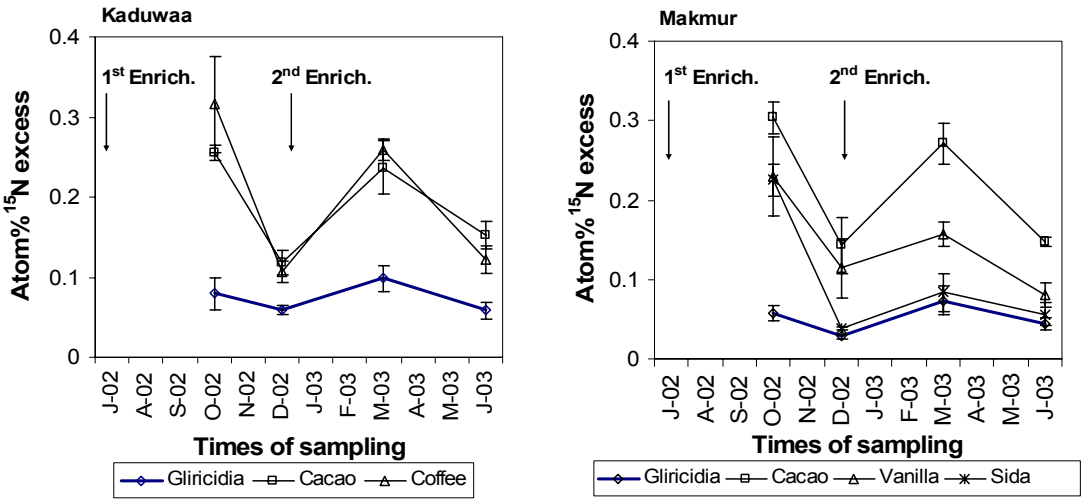

Figure 4.4: Atom% ^{15}N excess in leaves of fixing and reference plants at different times of sampling in Kaduwaa and Makmur (n=5; bars represent standard error of means)

In Kaduwaa, 12 weeks after the enrichment (Oct-02 and Mar-03), the atom% ^{15}N excess in the leaves of the reference plants increased to 0.26 and 0.24, and to 0.32 and 0.26 % for cacao and coffee at the 1st and 2nd enrichment, respectively. The values for the leaves of the fixing plant increased only slightly to 0.079 and 0.099 %, respectively. Subsequently, the atom% ^{15}N excess decreased proportionally (24 weeks after the enrichment, Dec-02 and Jun-03). The highest decrease was found in cacao (0.12 and 0.15 %) and coffee (0.10 and 0.12 %) at the 1st and 2nd enrichment. The lower decrease in the atom% ^{15}N excess of Gliricidia compared to the reference plants was attributed to a limited absorption of soil N by Gliricidia. In Makmur, the trend in Gliricidia, cacao, vanilla and sida was similar to that in Kaduwaa. Only sida showed a different pattern, which was close to that of the fixing plants. While Gliricidia leaves showed higher $\%N_{tot}$ than the reference plants, the $\%N_{tot}$ of cacao and Gliricidia in Kaduwaa were almost the same (Figure 4.5).

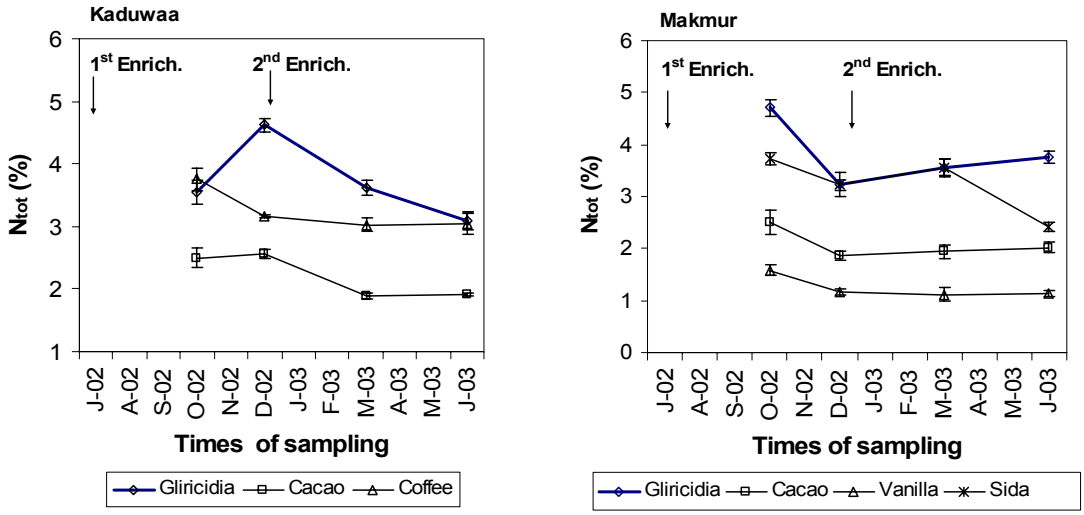

Figure 4.5: Proportion of N total (%N_{tot}) in leaves of fixing and reference plants at different times of sampling in Kaduwaa and Makmur (n=5; bars represent standard error of means)

The atom% ^{15}N excess in Gliricidia litter did not differ between samplings at both sites, while the values of reference plants varied significantly depending on plant species ($P<0.01$, see also Appendix 8). In Kaduwaa, the atom% ^{15}N excess in litter of coffee was higher than in that of cacao, and increased with time. In Makmur this trend followed the level of atom% ^{15}N excess in the soil, which increased in Oct-02 and Mar-03 and decreased in Dec-02 and Jun-03. In Kaduwaa, the %N_{tot} in the litter was higher in the fixing plant than in the reference plants, while in Makmur an opposite trend was observed.

4.1.3 Proportion of N derived from atmospheric N_2 (%Ndfa) of Gliricidia estimated with ^{15}NEM

The %Ndfa of Gliricidia differed depending on the plant organ used in the calculation. Using the leaves to calculate the %Ndfa of Gliricidia resulted in 57.3±4.2 % (45-69) with cacao and 52.8±5.7 % (39-68) with coffee as the reference plants. The reference plants did not turn out to influence the predictions of %Ndfa in Kaduwaa (Figure 4.6). This was not true in Makmur (Figure 4.7). Here, the mean %Ndfa of Gliricidia varied significantly depending on the reference plant used. Cacao resulted in the highest %Ndfa estimate. The mean %Ndfa of Gliricidia was 56.1±4.7 (44-63), 45.2±5.8 (41-51) and 29.0±5.1 % (18-55) with cacao, vanilla and sida, respectively, as reference plants. The low %Ndfa values for Gliricidia with sida as the reference plant may have been due

to the rapid decline of plant-available soil ^{15}N in the top soil layer (0-30 cm) where roots of sida mainly prevailed. Gliricidia and cacao have deeper roots than sida, hence, these plants may take up plant-available soil ^{15}N in deeper soil layers.

In Kaduwaa, the highest %Ndfa of Gliricidia was found in Oct-02, which was significantly higher than in Dec-02 and Jun-03 but not higher than in Mar-03 (Figure 4.6). However, in Makmur, the estimate was not affected by time of sampling (Figure 4.7).

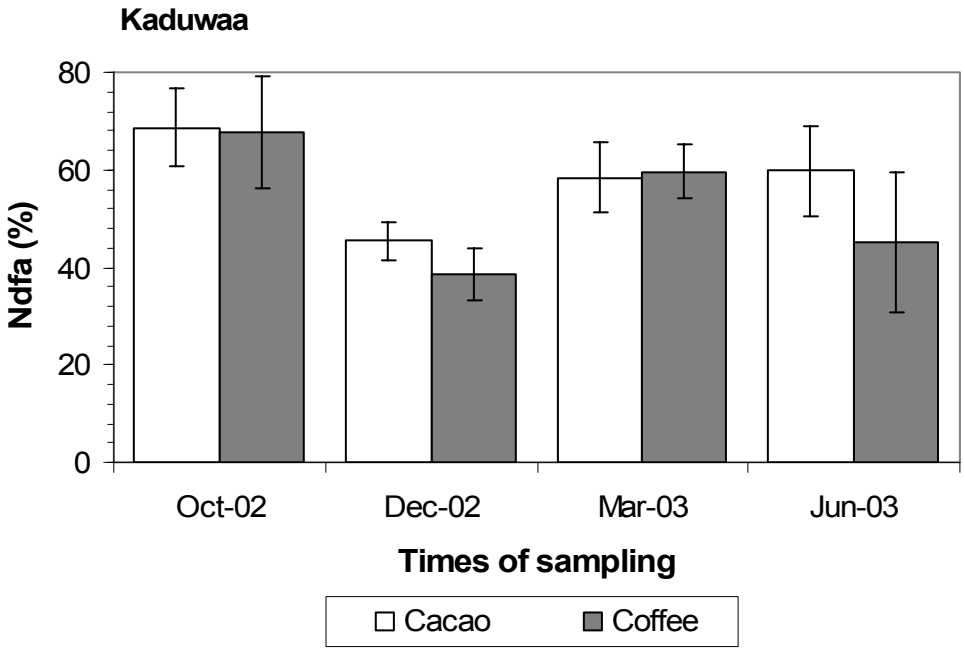

Figure 4.6: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using atom% ^{15}N excess in leaves with cacao and coffee as reference plants in Kaduwaa (n=5, bars represent standard error of means)

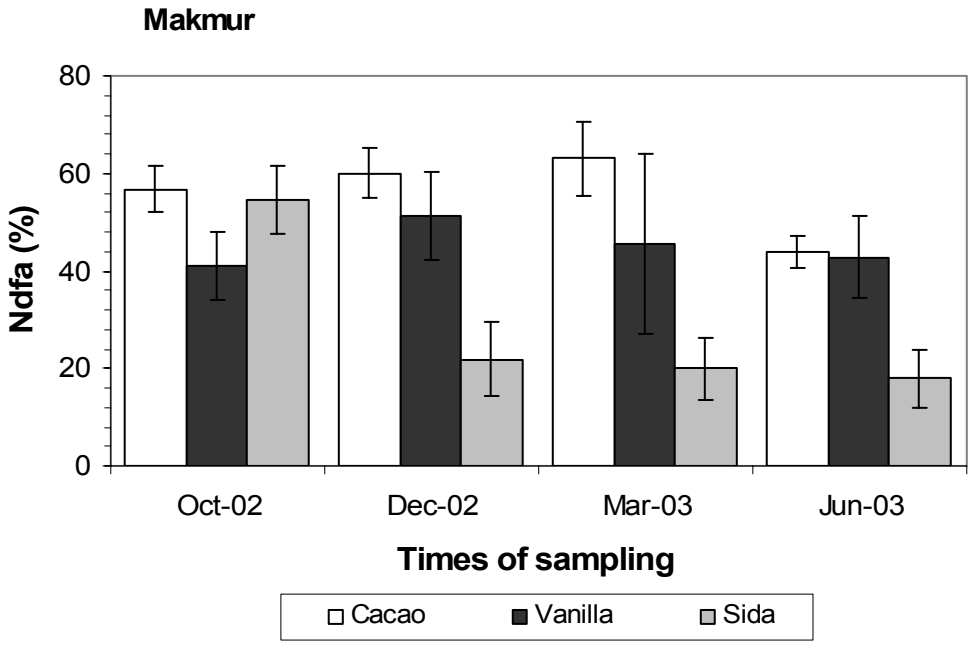

Figure 4.7: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using atom% ^{15}N excess in leaves with cacao, vanilla and sida as reference plants in Makmur (n=5, bars represent standard error of means)

The %Ndfa values for Gliricidia are in agreement with estimates with the same method in other cropping systems, e.g., in a Gliricidia monoculture in Africa (41-43 %; Sanginga et al. 1994), Australia (49-87 %; Peoples et al. 1996), in a hedgerow cropping system in Indonesia (55 %; Hairiah et al. 2000), or in an alley cropping system in Sri Lanka (55 %; Liyanage et al. 1994).

The %Ndfa of Gliricidia in Kaduwaa based on twigs resulted in 52.2±10.1 % with cacao and 58.1±8.9 % with coffee as reference plants. In Makmur, this was 70.6±3.6 % with cacao and 58.6±6.5 % with sida (Figure 4.8). The %Ndfa in Makmur with cacao as reference plant was 18 % higher than in Kaduwaa, while the estimates were similar with coffee and sida as reference plants, suggesting that it was rather the site that affected the %Ndfa estimate. This may be caused by different management practices and plant conditions such as pruning and plant densities.

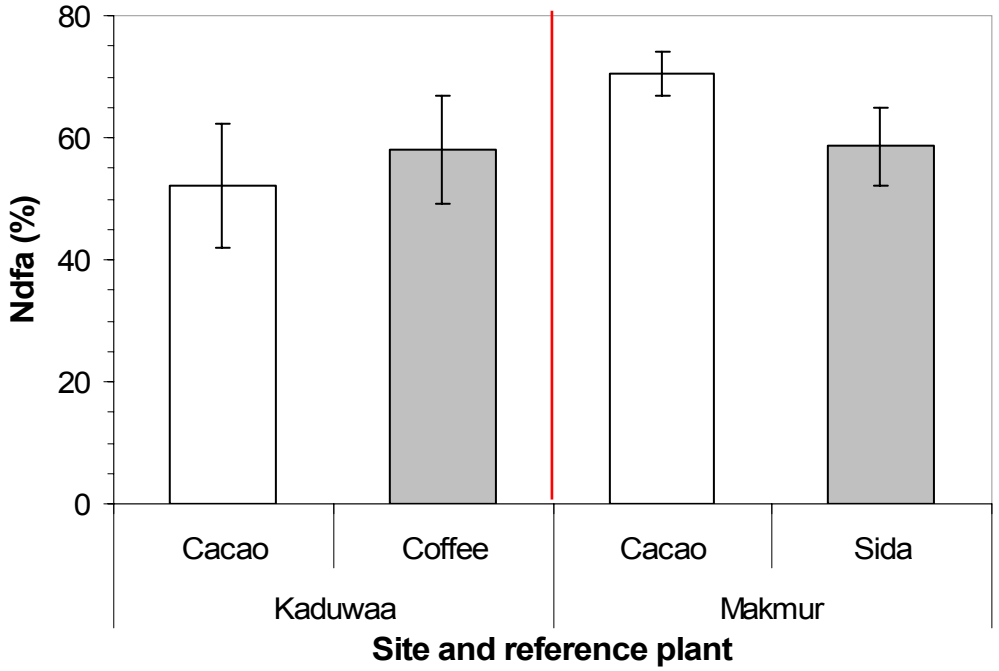

Figure 4.8: Proportion of N derived from atmospheric N_2 (%Ndfa) of Gliricidia in Oct-02 using atom% ^{15}N excess in twigs with cacao, coffee and sida as reference plants in Kaduwaa and Makmur (n=5, bars represent standard error of means)

Finally, when using the litterfall for calculating the %Ndfa of Gliricidia, the %Ndfa was only affected by the reference plants in Kaduwaa ($P<0.01$; Appendix 9). Coffee (61.9±4.1 %) resulted in higher %Ndfa values for Gliricidia than cacao (40.8±5.2 %, $P<0.01$). Though time of sampling did not affect the %Ndfa value for Gliricidia at both sites, the trend differed with time of sampling (Figure 4.9). In Kaduwaa, the %Ndfa of Gliricidia declined from Oct-02 to Mar-03 and increased in Jun-03. In Makmur, it followed the atom% ^{15}N excess of the soil, where in Dec-02 and Jun-03 the %Ndfa of Gliricidia was lower than that in Oct-02 and Mar-03.

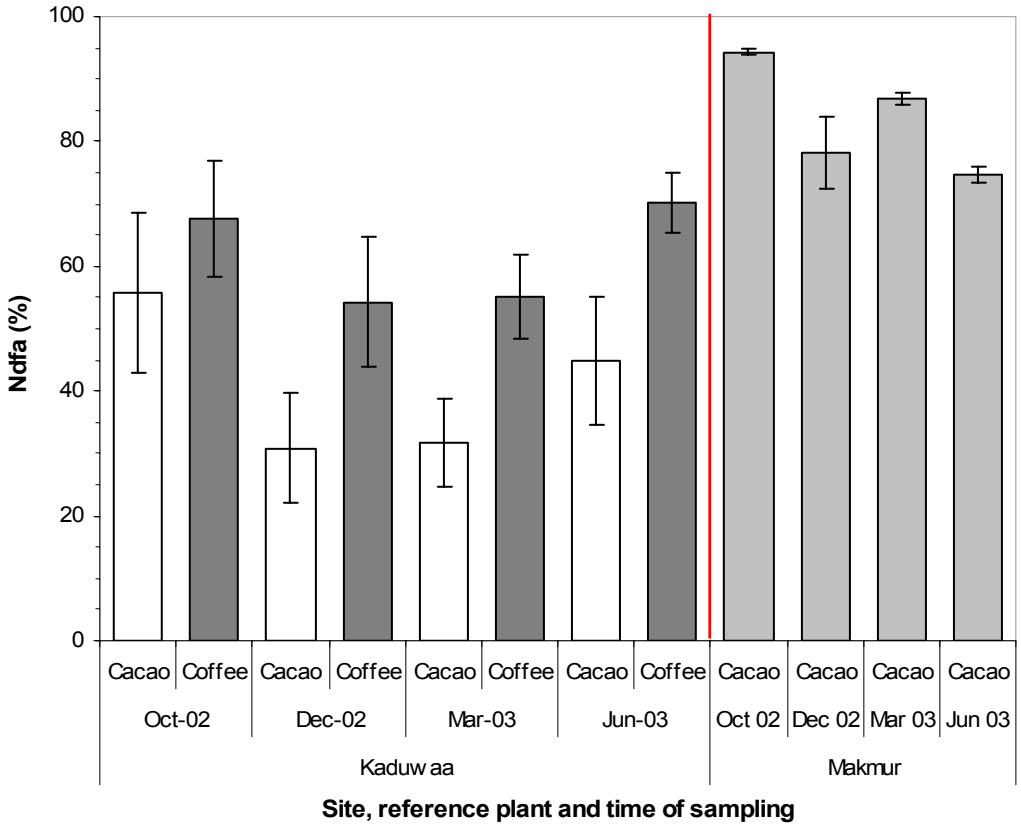

Figure 4.9: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using atom% ^{15}N excess in litter with cacao and coffee as reference plants in Kaduwaa and Makmur (n=5, bars represent standard error of means)

4.2 Nitrogen-15 natural abundance method

4.2.1 The $\delta^{15}N$ value and $\%N_{tot}$ in soil

Vertical and temporal variations of the $\delta^{15}N$ value of total soil N

The $\delta^{15}N$ value of total soil N as well as $\%N_{tot}$ varied significantly ($P<0.01$) with soil depth and time of sampling. There was an interaction between soil depth and time of sampling. This significantly affected the $\delta^{15}N$ value of total soil N at both sites ($P<0.01$; Appendix 10).

The mean $\delta^{15}N$ value of total soil N over all depths in Kaduwaa (8.16±0.20) was 0.75 ‰ higher than in Makmur (7.31±0.19; Figure 4.10). The pattern of the $\delta^{15}N$ values of total soil N at both sites was almost the same and showed little variation in deeper layers (30-150 cm); the top layer (0-30 cm) was less enriched, suggesting dilution by atmospheric N_2. In Kaduwaa, the $\delta^{15}N$ value of total soil N in 30-150 cm depth was around 8.5 ‰. It was significantly higher ($P<0.01$) than that in the top layer

(0-30 cm). In Makmur, the vertical distribution pattern was similar to that in Kaduwaa. The $\delta^{15}N$ value of total soil N in 30-50 cm depth was significantly higher than that in 0-10 cm depth. As expected and contrasting the $\delta^{15}N$ value of total soil N, the $\%N_{tot}$ in the soil at both sites decreased gradually with soil depth (Figure 4.10).

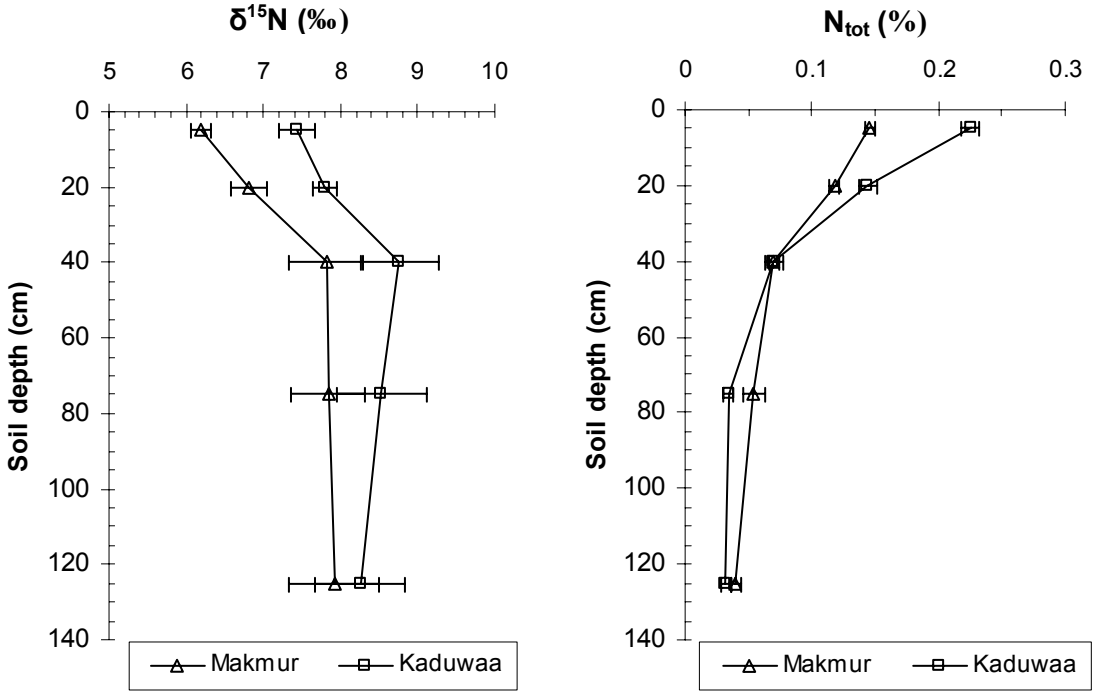

Figure 4.10: Mean $\delta^{15}N$ value of total soil N and $\%N_{tot}$ in soil at different depths in Kaduwaa and Makmur (n=25, bars represent standard error of means)

A relative depletion of the $\delta^{15}N$ value of total soil N in the top layer in comparison to the deeper layers was also reported by Ledgard et al. (1984) in improved pasture soils in Australia, in agroforestry systems in Kenya (Gathumbi et al. 2002) and in hedgerow agroforestry in Indonesia (Cadisch et al. 2000). An increase in the $\delta^{15}N$ value of total soil N with soil depth was also reported by other researchers (Mariotti et al. 1980; Tiessen et al. 1984; Nadelhofer and Fry 1988). This could be caused by plant litter, which tends to show a lower $\delta^{15}N$ value than that of the soil N pool from which the plants derived their N (Mariotti et al. 1980; Boddey et al. 2000). The natural variations in the $\delta^{15}N$ value of soil not only reflect the fractionation of the soil-N pool during microbial N transformation, but also different N processes (Piccolo et al. 1996). The accumulation of organic N and its transformation are the main processes

responsible for the variations in the $\delta^{15}N$ value of total surface soil N (Ledgard et al. 1984). In addition, fractionation of N during volatilization, mineralization, denitrification and leaching can also cause ^{15}N enrichment of residual N (Mariotti et al. 1980; Turner et al. 1983).

At both sites in three out of five times of sampling, i.e., in Jul-02, Mar-03 and Jun-03 in Kaduwaa and in Oct-02, Mar-03 and Jun-03 in Makmur, the vertical distribution of the $\delta^{15}N$ values of total soil N was homogenous (Figure 4.11). The remaining time the $\delta^{15}N$ value of total soil N varied significantly with soil depth ($P<0.01$). In Kaduwaa, the highest value was found in Oct-02 at 10-30 cm, which was significantly higher than the $\delta^{15}N$ values below this depth. Three months later, in Dec-02, the highest values were found at 50-100 cm. Similar trends were observed in Makmur in Jul-02 and Dec-02.

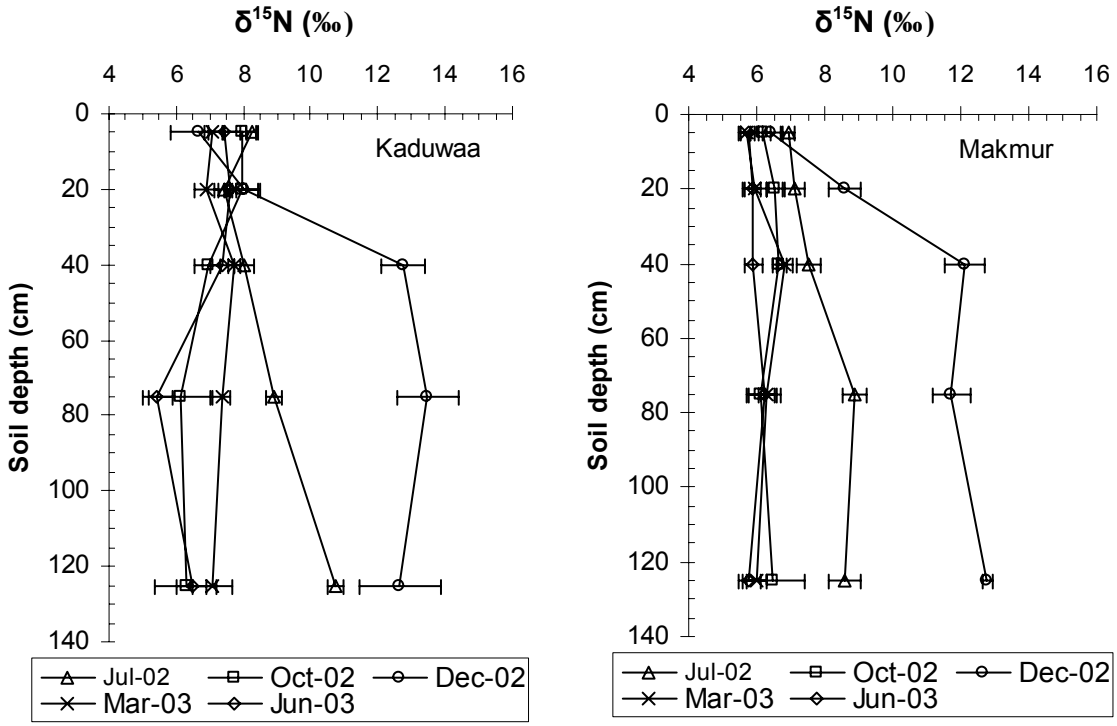

Figure 4.11: Mean $\delta^{15}N$ value of total soil N in soil at different depths and times of sampling in Kaduwaa and Makmur (n=5, bar represent standard error of means)

The higher $\delta^{15}N$ values of total soil N in Dec-02 at both sites was mainly caused by increasing $\delta^{15}N$ values in the lower soil layer (30-100 cm, Figure 4.11), where they increased from 6 to 12 ‰. According to Boddey et al. (2000), this is

uncommon. They state that short-term or annual changes of the $\delta^{15}N$ value of total soil are extremely unlikely in natural or agricultural sites owing to the recalcitrant nature of the majority of soil-organic N. However, six weeks before the measurement, there was a high precipitation (> 150 mm mo^{-1}) following a dry period of three months. Plant-available soil ^{15}N in the form of nitrate, which is more mobile than ammonium, may have been washed out to lower depths. A seasonal value of plant-available soil ^{15}N in the soil due to variation in the precipitation was also reported in hedgerow intercropping system in Indonesia by Hairiah et al. (2000).

Plant-available soil ^{15}N with depth

The $\delta^{15}N$ value of plant-available soil ^{15}N was significantly affected by site and soil depth (Table 4.5; Appendix 11). The mean $\delta^{15}N$ value of plant-available soil ^{15}N over depth in Kaduwaa (5.52±0.16) was higher than in Makmur (4.75±0.12). However, when the data were separated according to site, it was found that only in Kaduwaa was the $\delta^{15}N$ value of plant-available soil ^{15}N affected by depth. Though these differences are small, they could be a source of error in the estimation of the %Ndfa of Gliricidia, especially when the fixing and reference plants absorb different sources of N in the soil, which could lead to the over- or underestimation of the %Ndfa of fixing plants.

Table 4.5: Mean $\delta^{15}N$ value of plant-available soil N at different depths in Kaduwaa and Makmur

Depth (cm)	$\delta^{15}N$ value of mineral soil N[1](‰)	
	Kaduwaa	Makmur
0-10	5.69 (0.42) ab	4.48 (0.27)
10-30	6.28 (0.27) b	5.24 (0.09)
30-50	5.66 (0.22) ab	4.88 (0.39)
50-100	5.02 (0.14) a	4.65 (0.16)
100-150	4.96 (0.37) a	4.52 (0.25)
Mean	5.52 (0.16)	4.75 (0.19)
LSD (p = 0.05)	0.86*	0.72^{ns}

[1]Determined by ^{15}N analysis of total N of shoot of rice grown for 45 days in soil taken from the respective depths; values within one column followed by the same letter are not significantly different at P < 0.05; n=5; values in bracket represent standard error of means

Mineral ^{15}N and N in soil

Mineral ^{15}N and N in the soil of the natural abundance plots at different depths at the end of the experiment was analyzed. The $\delta^{15}N$ value of $^{15}NH_4^+$ and $^{15}NO_3^-$ in the soil at

both sites was not affected by depth (Table 4.6; Appendix 12). At both sites, the $\delta^{15}N$ value of $^{15}NH_4^+$ was much lower than that of $^{15}NO_3^-$. However, these results (especially for the $^{15}NH_4^+$) should be considered with caution. Some samples with an amount lower than 10 µg N showed higher variation. The $\delta^{15}N$ value of $^{15}NH_4^+$ and $^{15}NO_3^-$ was not affected by depth in Jun-03. This is not surprising, since the $\delta^{15}N$ value of total soil N was not affected by depth either (Figure 4.11). However, this was not the same in Dec-02, when the $\delta^{15}N$ value of total soil N at both sites was significantly affected by depth. Unfortunately, due to problems with the ^{15}N diffusion method analysis, the soil samples were not analyzed. The mineral soil NH_4^+ and NO_3^- concentrations in the soil decreased significantly with depth (Table 4.7; Appendix 12).

Table 4.6: The $\delta^{15}N$ value of mineral soil N in soil of natural abundance plot in Kaduwaa and Makmur at different depths determined at end of experiment (Jun-03)

Soil depth	Kaduwaa		Makmur	
	$^{15}NH_4^+$	$^{15}NO_3^-$	$^{15}NH_4^+$	$^{15}NO_3^-$
(cm)	(‰)	(‰)	(‰)	(‰)
0-10	1.96 (2.47)	3.08 (2.38)	-1.05 (1.05)	3.56 (0.26)
10-30	1.90 (0.37)	5.81 (2.38)	-2.06 (0.59)	3.85 (1.67)
30-50	0.45 (1.21)	4.43 (3.74)	-5.01 (0.21)	6.95 (1.32)
50-100	4.54 (2.69)	3.90 (0.51)	-3.32 (2.72)	4.05 (1.34)
100-150	3.58 (1.29)	4.37 (1.77)	-2.64 (0.04)	7.32 (2.82)
LSD (p= 0.05)	ns	ns	ns	ns

ns=not significant; n=3; values in parentheses represent standard error of means

Table 4.7: Mineral N[§] in soil of natural abundance plot at different depths determined at end of experiment (Jun-03)

Soil depth	NH_4^+	NO_3^-
(cm)	mg kg^{-1}	mg kg^{-1}
0-10	9.4 (0.9) d	8.2 (3.4) b
10-30	5.7 (0.6) c	2.9 (0.9) ab
30-50	4.1 (0.5) b	1.5 (0.6) a
50-100	1.3 (0.1) a	0.9[#]
100-150	1.8 (0.4) a	1.6[#]
LSD (p= 0.05)	1.4	5.6

[§]*Data from Kaduwaa and Makmur site were merged, since site did not affect plant-available soil N;* [#]*only detected in one replication; n.d. not detected; values within one column followed by the same letter are not significantly different at p < 0.05; n=6; values in parentheses represent standard error of means*

4.2.2 The $\delta^{15}N$ value and $\%N_{tot}$ in plants

The $\delta^{15}N$ values and $\%N_{tot}$ in the leaves, twigs and litter in Kaduwaa and Makmur were affected by plant species and time of sampling. There was also an interaction between plant species and time of sampling. This significantly affected $\delta^{15}N$ value and $\%N_{tot}$ in the leaves, twigs and litter at both sites (Appendix 13 and 14).

Variations among plants and plant parts

The $\delta^{15}N$ values in the leaves of reference plants in Kaduwaa were 1.6-1.8 ‰ higher than those of the fixing plant, with overall means and standard errors over time of 3.46±0.23, 5.32±0.25 and 5.71±0.37 ‰ for Gliricidia, cacao and coffee, respectively ($P<0.01$; Table 4.8). In Makmur, these were 1.1-3.0 ‰ higher than those of Gliricidia, with overall means and standard errors of $\delta^{15}N$ value over time of 2.31±0.23, 3.49±0.21, 6.01±0.47 and 3.68±0.27 ‰ for Gliricidia, cacao, vanilla and sida, respectively ($P<0.01$). The $\delta^{15}N$ values in the twigs and litter at both sites showed similar trend to those in the leaves, whereas the $\delta^{15}N$ values in the reference plants were 1-2 ‰ and 1.5-2 ‰ higher than those in Gliricidia in Kaduwaa and Makmur, respectively ($P<0.01$).

Table 4.8: Mean $\delta^{15}N$ value in the leaves, twigs and litter of fixing and reference plants in Kaduwaa and Makmur

Plant	Leaves	Twigs	Litter
		$\delta^{15}N$ value (‰)	
		Kaduwaa	
Gliricidia	3.46±0.23 a	3.10±0.46 a	2.99±0.22 a
Cacao	5.32±0.25 b	4.31±0.28 b	4.42±0.17 b
Coffee	5.71±0.37 c	5.08±0.32 c	4.27±0.22 b
LSD (p=0.05)	0.36	0.76	0.33
		Makmur	
Gliricidia	2.31±0.23 a	0.68±0.27 a	2.26±0.32 a
Cacao	3.49±0.21 b	1.79±0.55 b	4.24±0.25 b
Vanilla	6.01±0.47 c	Nd	nd
Sida	3.68±0.27 b	3.53±0.42 c	nd
LSD (p=0.05)	0.60	0.62	0.55

Values within one column followed by the same letter are not significantly different at P < 0.05; nd = not determined; n=25; values in bracket represent standard error of the means

Branches or twigs of plants are generally less enriched in ^{15}N than leaves (Shearer and Kohl 1986; Virginia et al. 1989). Variations in the $\delta^{15}N$ values between plant parts could be the results of (i) ^{15}N discrimination during transport of ^{15}N within

plant parts, (ii) changes in the N_2 fixation ability during the development of the plant, and (iii) changes in the $\delta^{15}N$ value of the plant-available soil-N pool (Cadisch et al. 2000).

The $\delta^{15}N$ values of fixing and reference plants in this study are within the range of fixing shrubs and trees and reference plants found in other studies (Shearer and Kohl 1986; Yoneyama et al. 1993; Ladha et al. 1993; Gathumbi et al. 2002). Higher $\delta^{15}N$ values of reference plants compared to fixing plants were also found by Ladha et al. (1993) in hedgerows of *Cassia spectabilis* (non-fixing plant) and Gliricidia in all parts of the plants (leaves, stems and trunks). Lower values of fixing plants compared to those of reference plants and the $\delta^{15}N$ value of total soil N suggest that much of the N of the fixing plant has its origin in the atmospheric N_2, whereas that of reference plants is primarily derived from the soil (Delwiche and Steyn 1970).

The low variability in the $\delta^{15}N$ value of the different reference plants (cacao and coffee in Kaduwaa, and cacao and sida in Makmur) over time suggests uniform uptake of the $\delta^{15}N$ of plant-available soil N. This is also supported by the fact that though the $\delta^{15}N$ value of total soil N over time varied significantly over the whole depth (0-150 cm), it showed only little variation in the 0-50 cm depth (Table 4.5), which is the main rooting zone of cacao, coffee and sida. However, this did not apply to vanilla. Though the $\delta^{15}N$ value of total soil N under the canopy of fixing and reference plants was not measured separately, it is assumed that litterfall deposited over time under the canopy of Gliricidia enriched the soil N pool more than under the canopy of the reference plants. As a consequence, the higher $\delta^{15}N$ value in vanilla than in the other reference plants could be due to the fact that vanilla uses Gliricidia as a supporting/climbing tree (grown always close to Gliricidia) and takes up slightly $\delta^{15}N$-enriched plant-available soil N.

As expected, the fixing plants had the highest $\%N_{tot}$ in all plant parts at both sites. The lowest $\%N_{tot}$ was measured in cacao in almost in all plant parts ($P<0.01$), except for Makmur, where the $\%N_{tot}$ was the lowest in the leaves of vanilla (Table 4.9).

Table 4.9: Proportion of N total (%N_{tot}) in leaves, twigs and litter of fixing and reference plants in Kaduwaa and Makmur

Plant	Leaves	Twigs	Litter
		N_{tot} (%)	
		Kaduwaa	
Gliricidia	3.96±0.13 c	1.75±0.24 b	2.22±0.05 b
Cacao	2.16±0.08 a	0.90±0.05 a	1.32±0.05 a
Coffee	3.14±0.03 b	1.64±0.06 b	2.29±0.04 b
LSD (*p*=0.05)	0.17	0.15	0.10
		Makmur	
Gliricidia	3.76±0.14 d	1.60±0.06 c	1.72±0.09 b
Cacao	2.08±0.10 b	0.88±0.04 a	1.25±0.07 a
Vanilla	1.25±0.06 a	Nd	nd
Sida	3.23±0.14 c	1.66±0.31 b	nd
LSD (*p*=0.05)	0.15	0.19	0.09

Values within one column followed by the same letter are not significantly different at P < 0.05; nd = not determined; n=25; values in bracket represent standard error of the means

Variations in different plant parts and times of sampling

In Kaduwaa, the $\delta^{15}N$ value in the leaves of Gliricidia increased significantly (P<0.01) from Jul-02 to Oct-02, decreased significantly (P<0.05) to Mar-03 and slightly increased in the subsequent harvest (Figure 4.12). For the reference plants, the trend was similar, with a significant decrease in the $\delta^{15}N$ value from Oct-02 to Mar-03 followed by an increase from Mar-03 to Jun-03 (significant for cacao only). In Makmur, the trend in the leaves of Gliricidia, cacao and sida was similar to that in Kaduwaa. The $\delta^{15}N$ value in the leaves of vanilla, however, showed a different pattern with a steep increase from Jul-02 to Mar-03 and subsequent decrease to Jun-03 (P<0.01).

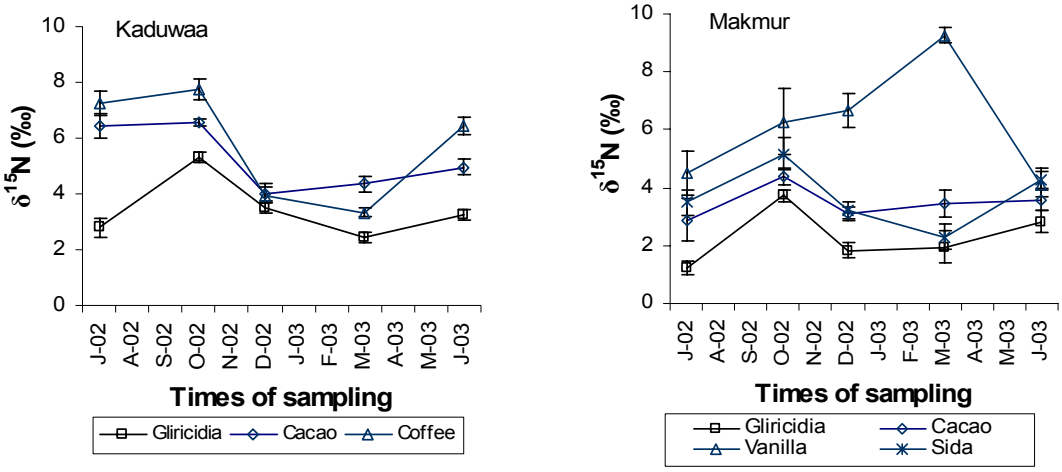

Figure 4.12: Mean $\delta^{15}N$ value in leaves of fixing and reference plants at different times of sampling in Kaduwaa and Makmur (n=5; bars represent standard error of the means)

As expected, the $\%N_{tot}$ in the leaves of the fixing plant was significantly higher than in the reference plants at both sites ($P<0.01$). However, this was not true in Dec-02, when the $\%N_{tot}$ in the leaves of sida had increased drastically and was slightly higher than that of Gliricidia (Figure 4.13).

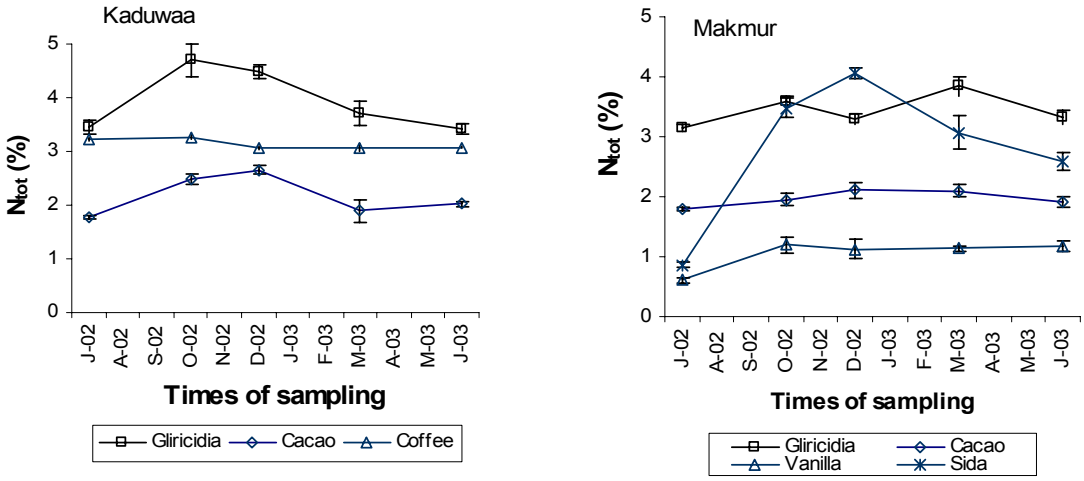

Figure 4.13: Mean $\%N_{tot}$ in leaves of fixing and reference plants at different times of sampling in Kaduwaa (n=5; bars represent standard error of the means)

The seasonal patterns of the $\delta^{15}N$ values in the plants provide important information about changes in the relative contribution of soil and atmospheric sources of N to the plants (Virginia et al. 1988). Sprent (1972) observed that nodule activity is reduced in response to dry and water-logged conditions. He observed maximum N_2

fixation when soils were near field capacity. Lower N_2-fixing activity due to lower precipitation was also reported by Bremer and van Kessel (1990). This also seems to be true in the present study. The results show that substantial differences exist between and within fixing and reference plants across the five times of sampling in response to rainfall amounts. In Oct-02, after two months of lower precipitation, the $\delta^{15}N$ value of the reference plants still remained constant or only slightly increased as compared to the values measured in Jul-02, but the $\delta^{15}N$ value of the fixing plant increased substantially, suggesting that it took up more N from plant-available soil N as a result of reduced of N_2 fixation activity. However, the Dec-02 values do not support this argumentation: At that time, the $\delta^{15}N$ value of fixing and reference plants decreased substantially, even though starting at the beginning of Nov-02 until mid Dec-02, the precipitation at both sites had almost doubled. The decline in the $\delta^{15}N$ value of the fixing plant may have been caused by increasing N_2 fixation activities by Gliricidia, hence more N was absorbed from the atmospheric N_2. or may be have been the result of lower $\delta^{15}N$ values of plant-available soil N in the rooting zone, as this had been washed out to lower depths. Bergensen et al. (1989) state that the difference in the $\delta^{15}N$ values between fixing and reference plants may be caused by an uptake of soil N differing in $\delta^{15}N$ values. If the $\delta^{15}N$ value of reference plants is a true measure of the $\delta^{15}N$ value of plant-available soil N (Ledgard et al. 1984; Peoples et al. 1989), the variability in the $\delta^{15}N$ value of reference plants is likely due to the variability in the $\delta^{15}N$ value of plant-available soil N with time and/or depth. Thus, a lower $\delta^{15}N$ value of the reference plant reflects a lower $\delta^{15}N$ value of plant-available soil N. In the considered period (Oct-02 to Dec-02), the lower value was caused by the flush of plant-available soil ^{15}N to deeper depths, as can be seen by a significant increase in the $\delta^{15}N$ value of total soil N at deeper depths during this period (Figure 4.11). Bremer and van Kessel (1990) explained two scenarios that may affect the difference in the $\delta^{15}N$ of soil derived N with time and depth between fixing and reference plants:1) soil N is obtained at different times and the $\delta^{15}N$ of plant-available soil N varies with time, or 2) soil N is obtained from different depths and the $\delta^{15}N$ of plant-available soil N varies with depth. The results in this study reflect both scenarios, i.e., the $\delta^{15}N$ value of total soil N varies not only with depth but also with time.

Variations of the $\delta^{15}N$ values among reference plants may also be affected by differences in plant rooting patterns and by ^{15}N fractionation between plant species. In addition, inconsistency in sampling (age and nature of plant samples) may also result in different $\delta^{15}N$ values. Thielen-Klinge (1997) observed that even in the same species, the $\delta^{15}N$ value in the young leaves varies considerably compared with that in the old leaves. Gathumbi et al. (2002) reported that the $\delta^{15}N$ value of reference plants is influenced by rooting depth as well as by differences between species. Therefore, even with a uniform vertical distribution of ^{15}N in soils, the right choice of reference plants is crucial. The inconsistency of the $\delta^{15}N$ value of reference plants over time in this study emphasizes that the %Ndfa estimate should not only rely on one 'appropriate' reference plant, but best on several reference plants (Boddey et al. 2000).

The trend of the $\delta^{15}N$ value and $\%N_{tot}$ in the twigs and litter of fixing and reference plants at both sites showed similar patterns to that observed in the leaves (Appendix 15 and 16). As expected, $\%N_{tot}$ was higher in the fixing than in the reference plants. Both results are commonly observed in agroforestry systems (Ladha et al. 1993; Gathumbi et al. 2002).

4.2.3 Nitrogen-15 discrimination (*B*-value)

The $\delta^{15}N$ values and the accumulation of N in Gliricidia grown in pure sand under greenhouse conditions were significantly different ($P<0.01$) among plant parts at each time of sampling (Appendix 17). The lowest $\delta^{15}N$ value was found in the leaves, with -0.21±0.06, -1.47±0.36 and -0.45±0.15 ‰ at 12, 24 and 36 (week after planting (WAP), respectively (Figure 4.14). The highest $\delta^{15}N$ value was found in the nodules, with 8.73±0.14 ‰, 10.96±0.51 and 8.48±0.21 ‰ at 12, 24 and 36 WAP, respectively. Though the highest $\%N_{tot}$ was found in the nodules (4.86, 4.13 and 4.35 % at 12, 24 and 36 WAP, respectively), the lowest $\%N_{tot}$ was found in the stems (1.08, 1.67 and 1.44 % at 12, 24 and 36 WAP, respectively). The mean of the total N accumulated in the whole plant increased from 0.13 g N $plant^{-1}$ at 12 WAP to 1.01 g N $plant^{-1}$ 36 WAP. Nitrogen accumulated in the leaves and roots was higher than in the other plant parts ($P<0.01$). The accumulation of N in the different plant parts was also affected by the age of the plants. At 12 and 36 WAP, more N was found in the leaves and roots than in other parts. The proportion of N belowground (roots and nodules) compared to aboveground

(shoots) at each time of sampling was 42, 41 and 35 %. This shows that belowground biomass plays a major role in contributing N to the soil N pool in the field. Since only leaves, twigs and litter samples were taken for estimating the %Ndfa of Gliricidia, it is proposed to relate the *B*-value also to merely these components sampled 36 WAP. Thus the *B*-value used in this study is 0.41±0.07 ‰.

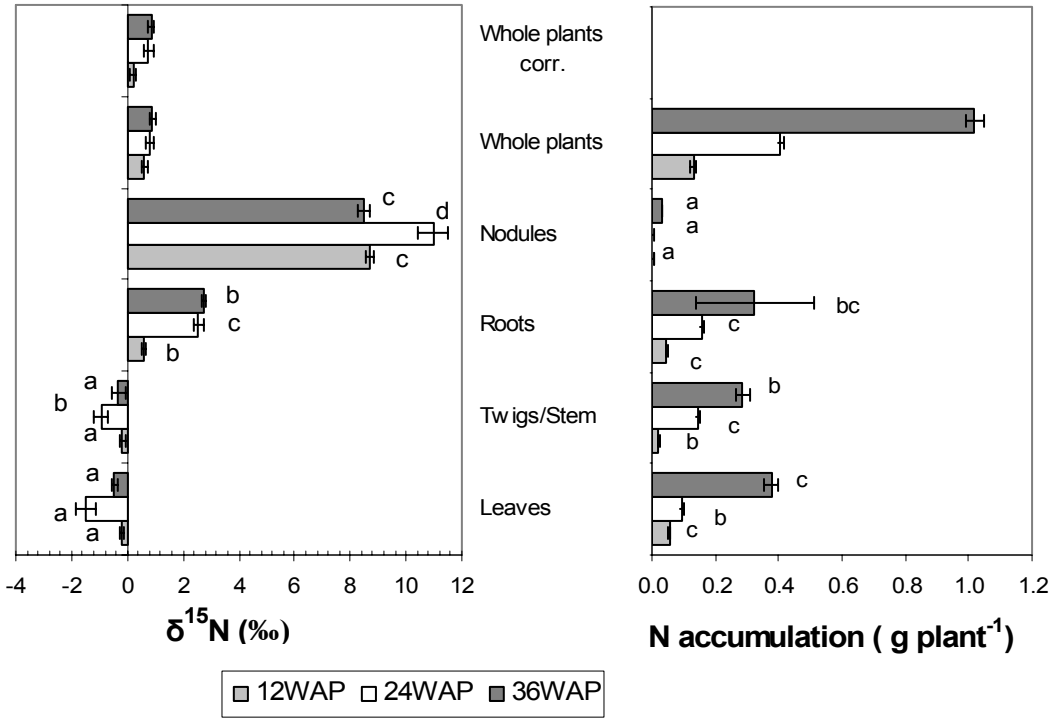

Figure 4.14: Mean $\delta^{15}N$ value and total N accumulation in Gliricidia grown in pure sand and irrigated with N-free solution under greenhouse conditions; whole plant corr. was corrected for seed ^{15}N; bars of the same pattern with different letters are significantly different at $P<0.05$; n=10, means and standard error of the means, WAP = weeks after planting

Negative $\delta^{15}N$ values in the shoots and positive $\delta^{15}N$ values in the roots and nodules have also been observed in a range of tree legumes by Gathumbi et al. (2002). The $\delta^{15}N$ values in the shoots (i.e., leaves, twigs and stems) of Gliricidia in their study ranged between -0.21 and -0.61 ‰ within the first 36 weeks after planting. Ladha et al. (1993) and Hairiah et al. (2000) observed slightly lower $\delta^{15}N$ values in the shoots of the same plant species grown in the Philippines (-1.45 ‰) and in Indonesia (-1.11 ‰), respectively. Besides the time of harvest, the type of innoculant also affected the $\delta^{15}N$ value of the fixing plant. Cadisch et al. (1993) demonstrated in-situ that a range of

rhizobial strains is likely to be involved in the legume-*Rhizobium* symbiosis. Steel et al. (1983) reported that ^{15}N isotope discrimination is strongly influenced by plant species, and the infecting rhizobial strain and the variations in the natural abundance of ^{15}N due to different strains can amount to as much as 2 ‰.

4.2.4 Proportion of N derived from atmospheric N_2 (%Ndfa) of Gliricidia estimated with ^{15}NNAM

The %Ndfa of Gliricidia estimated with the ^{15}NNAM differed depending on the plant organ used in the calculation. Using leaves to calculate the %Ndfa of Gliricidia, the reference plants affected the estimate in Makmur but not in Kaduwaa (Appendix 18). In Kaduwaa, the %Ndfa of Gliricidia was 30.9±4.4 % (11-53) with cacao and 33.8±3.9 % (10-58) with coffee as reference plants. In Makmur, values were 31.8±9.5 (14-50), 55.4±5.3 (29-68) and 36.9±7.3 % (26-58), with cacao, vanilla and sida, respectively, as reference plants. Thus, the %Ndfa of Gliricidia with cacao as a reference plant at both sites agreed within 1 %, while using coffee and sida led to values 3 to 6 % higher than with cacao. Vanilla as a reference plant did not match the %Ndfa estimate based on the other three reference plants and was 19 to 25 % higher.

The %Ndfa of Gliricidia determined with ^{15}NNAM in this study is lower than the estimate for the same crops in other cropping systems. In the Philippines, Ladha et al. (1993) reported %Ndfa values for Gliricidia in alley cropping with Senna as a reference plant between 52 and 64 %. In a Gliricidia monoculture in Australia, the values ranged from 56-89 % (Peoples et al. 1996). The results in this study are also lower than the 44-58 % Ndfa reported by Rowe et al. (1999) for Gliricidia in a hedgerow intercropping system with *Pheltophorum dasyrrachis* in Indonesia. They are only slightly lower than the 37 % reported by Hairiah et al. (2000) for Gliricidia in hedgerow trees with *Pheltophorum dasyrrachis* in Lampung Indonesia. Differences in the %Ndfa of Gliricidia may be caused by differences in rooting patterns and N uptake of fixing and reference plants, environmental factors such as soil moisture (precipitation), soil rhizobia, and plant-available soil N.

Time of sampling affected the %Ndfa estimate (Figure 4.15 and 4.16). The highest amounts at both sites were estimated in Jul-02 (55 to 58 %) and the lowest in Dec-02 (Kaduwaa, 10 %) and Oct-02 (Makmur, 26 %). In Kaduwaa, both reference

plants showed a similar pattern at each time of sampling. In Makmur, the %Ndfa estimate varied among reference plants at each time of sampling. Three out of five times, using vanilla as the reference plant, resulted in a %Ndfa of more than 60 %.

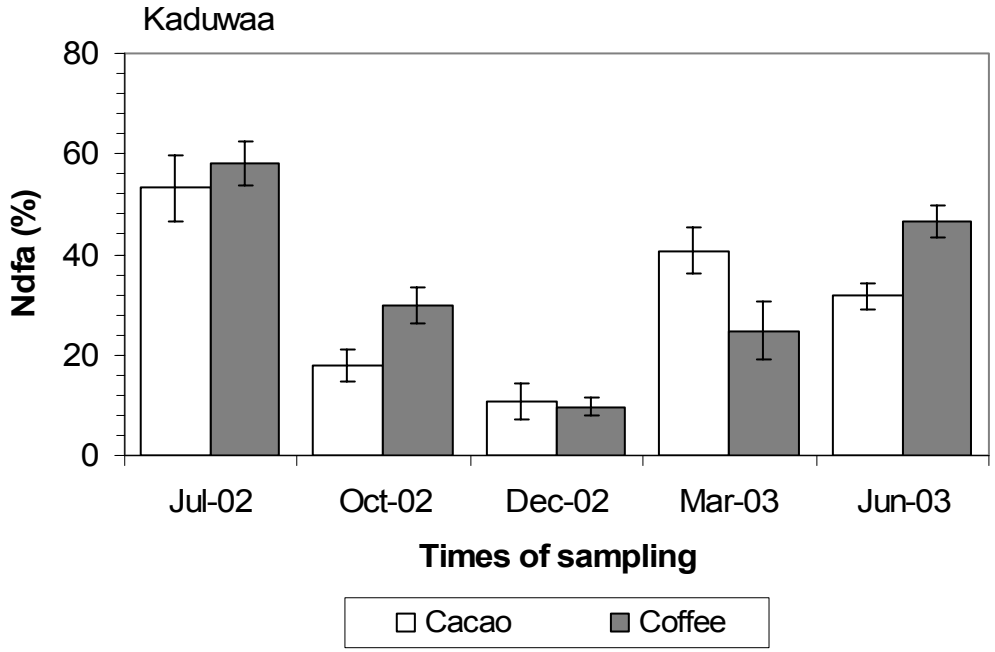

Figure 4.15: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using $\delta^{15}N$ value in leaves with cacao and coffee as reference plants in Kaduwaa (n=5, bars represent standard error of the means)

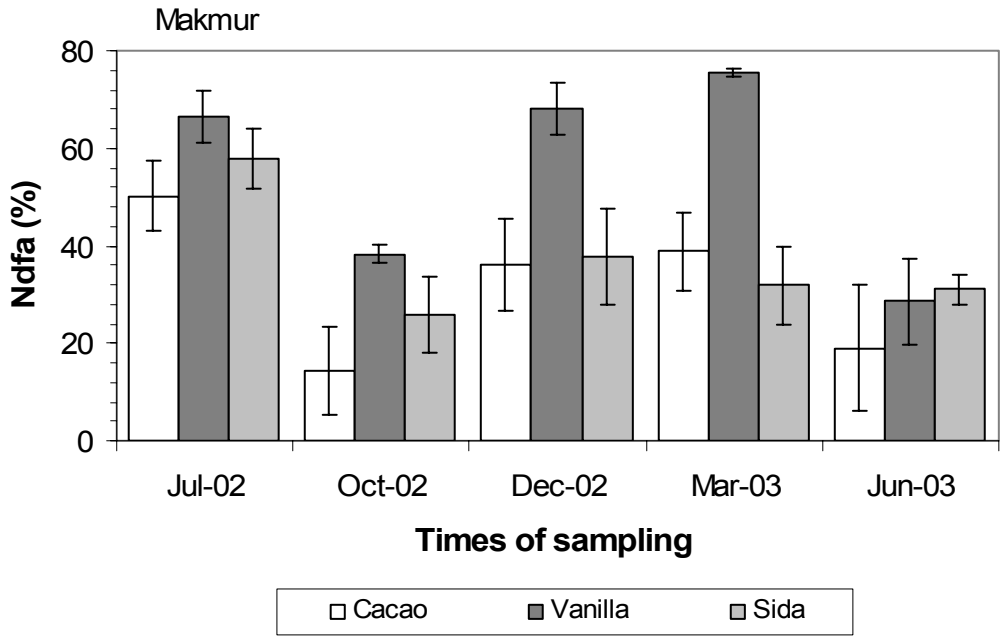

Figure 4.16: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using $\delta^{15}N$ value in leaves with cacao, vanilla and sida as reference plants in Makmur (n=5, bars represent standard error of the means)

Uncovich et al. (1994) suggest that the %Ndfa estimates are more dependent on the $\delta^{15}N$ value of the fixing plant than on the $\delta^{15}N$ value of the reference plants. However, Gathumbi et al. (2002) found that different $\delta^{15}N$ values of reference plants result in different %Ndfa estimates, suggesting that reference plants play also a significant role in estimating %Ndfa even when applying the ^{15}NNAM. Finally, and this is probably the most important reason for different %Ndfa estimates, is the increase in plant-available soil N, which is also known to reduce the %Ndfa of fixing plants. Van Kessel et al. (1994) showed that N_2 fixation decreases with time in *Leucaena leucocepala* hedgerows as a result of a recycling of fixed N.

Seasonal variations in the %Ndfa of Gliricidia have also been reported by Hairiah et al. (2000) in a hedgerow cropping system in Lampung Indonesia. They found that with the onset of rains the %Ndfa declined until December (dry season) but recovered toward the end of the rainy season (April and June).

Using the $\delta^{15}N$ value in the twigs to quantify the %Ndfa of Gliricidia, the %Ndfa estimate depended also on reference plant and time of sampling (Figure 4.17). In Kaduwaa, the %Ndfa of Gliricidia (mean of two sampling times) was 42.9±10.5 and

50.0±8.9 % with cacao and coffee, respectively, as reference plants. The %Ndfa estimate was slightly higher in Jul-02 (47 and 60 %) than in Oct-02 (39 and 40 %) with cacao and coffee, respectively. In Makmur, this was 48.5±13.9 and 70.5±6.5 % with cacao and sida, respectively, as reference plants. The %Ndfa of Gliricidia in Makmur with cacao was higher than in Kaduwaa.

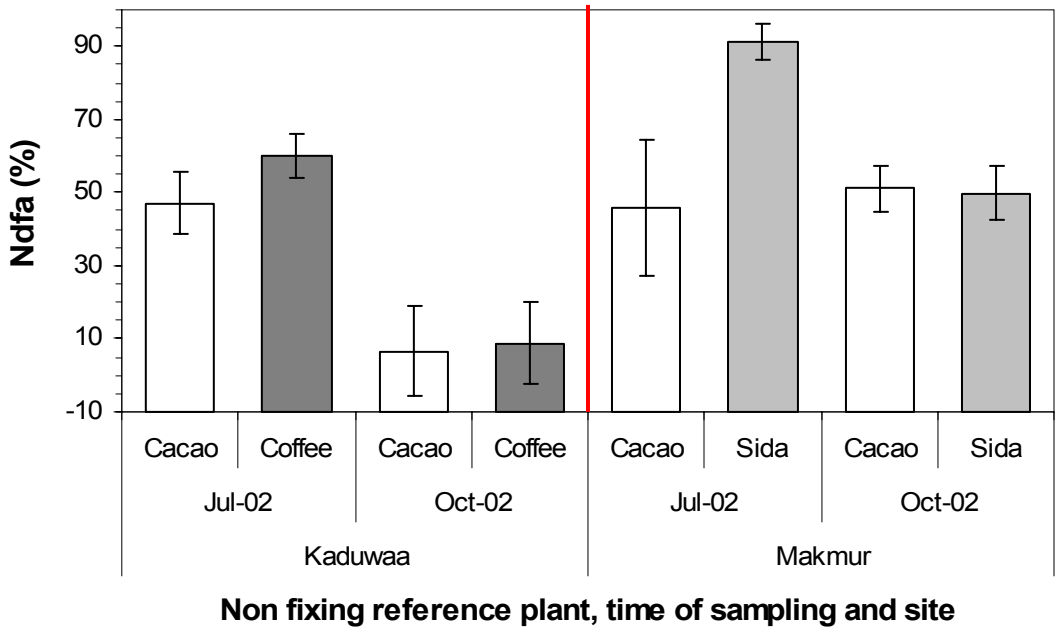

Figure 4.17: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia at different times of sampling using $\delta^{15}N$ value in twigs with cacao, coffee and sida as reference plants in Kaduwaa and Makmur (n=5, bars represent standard error of the means)

The %Ndfa of Gliricidia using the $\delta^{15}N$ value in the litter depended also on reference plant and time of sampling (Figure 4.18). In Kaduwaa, the %Ndfa was 28.3±6.2 and 26.8±8.3 % with cacao and coffee, respectively, as reference plants. In Makmur, with cacao as reference plant, it was slightly higher than in Kaduwaa that was 38.3±10.4 %.

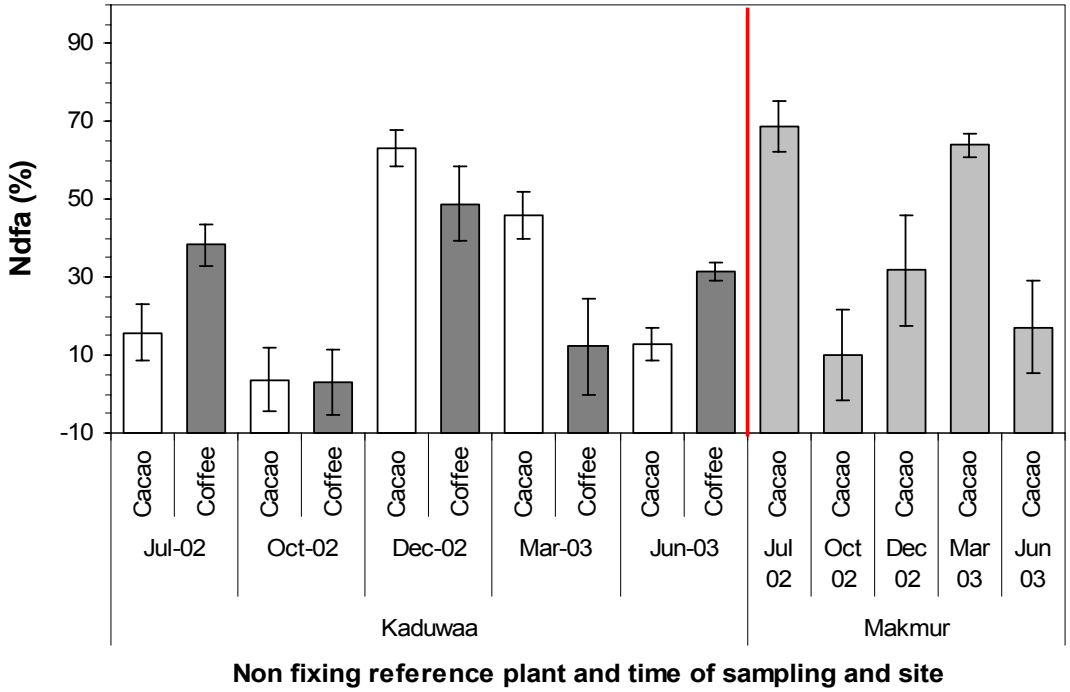

Figure 4.18: Proportion of N derived from the atmospheric N_2 (%Ndfa) of Gliricidia at different times of sampling using the $\delta^{15}N$ value in litter with cacao and coffee as reference plants in Kaduwaa and Makmur (n=5, bars represent standard error of the means)

4.3 Comparison of ^{15}NEM and ^{15}NNAM

The %Ndfa of Gliricidia using the atom% ^{15}N excess and $\delta^{15}N$ value in the leaves determined with ^{15}NEM and ^{15}NNAM differed significantly (*t*-Test; $P<0.01$) in cacao and coffee as the reference plants. This was 22 to 33 % higher with ^{15}NEM than with ^{15}NNAM. However, both methods did not differ significantly when vanilla and sida were used (Table 4.10). This similarity does not mean that the above reference plants are optimal both methods due to the higher %Ndfa of Gliricidia estimated with the ^{15}NNAM with vanilla as the reference plant compared to the lower %Ndfa estimate with the ^{15}NEM with sida as the reference plant. Furthermore, there is a strong variability in the %Ndfa values with both reference plants at each time of sampling. These results contrast to the findings of Hairiah et al. (2000) in hedgerow cropping systems with Gliricidia in Indonesia, and those of Peoples et al. (1996), who observed in a field monoculture of Gliricidia in Australia that both methods resulted in similar mean %Ndfa estimate. On the other hand, the results support the findings of Hariah et al.

(2000) that the ^{15}NEM results in higher %Ndfa estimates (by 18 % on average) than ^{15}NNAM.

Table 4.10: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia determined with ^{15}N enrichment method (^{15}NEM) and ^{15}N natural abundance method (^{15}NNAM) using atom% ^{15}N excess and δ^{15}N values in leaves at different times of sampling in Kaduwaa and Makmur

Site	Non-fixing reference plant	Time of sampling	%Ndfa		Probability (Paired *t*-Test)
			^{15}NEM	^{15}NNAM	
Kaduwaa	Cacao	Oct-02	68.7	17.9	$P < 0.01$
		Dec-02	44.9	14.4	$P < 0.05$
		Mar-03	56.4	40.0	ns
		Jun-03	56.5	31.1	ns
		Mean	57.3	26.2	$P < 0.01$
	Coffee	Oct-02	67.7	29.2	$P < 0.05$
		Dec-02	38.5	10.5	$P < 0.05$
		Mar-03	57.7	28.4	$P < 0.05$
		Jun-03	45.3	46.3	ns
		Mean	52.8	29.6	$P < 0.01$
Makmur	Cacao	Oct-02	56.7	20.4	$P < 0.01$
		Dec-02	61.0	29.8	$P < 0.01$
		Mar-03	63.1	43.9	$P < 0.05$
		Jun-03	43.5	24.9	ns
		Mean	56.1	26.2	$P < 0.01$
	Vanilla	Oct-02	41.0	33.5	ns
		Dec-02	51.3	64.0	ns
		Mar-03	45.4	75.7	ns
		Jun-03	42.9	19.7	ns
		Mean	45.1	46.4	ns
	Sida	Oct-02	54.5	23.3	$P < 0.01$
		Dec-02	18.9	29.9	ns
		Mar-03	18.2	25.2	ns
		Jun-03	17.9	29.9	ns
		Mean	29.0	26.9	ns

ns = not significant; n= 5

Time of sampling also plays a significant role in the accuracy of the %Ndfa estimate for Gliricidia with both methods. In only one (Jun-03) out of four times of sampling (Oct-02–Jun-03), did the %Ndfa estimates with the two methods and based on four different reference plants not differ significantly. Lack of agreement between both methods at different times of sampling was also observed by Hamilton et al. (1993) for the quantification of BNF of *Acacia sp*. within a natural *Eucalypt* forest. Only at

harvest, 16 months after planting, was a good agreement observed between both methods using *Poa sieberiana* and other 'opportunistic reference species'. They stated that this was due to (1) the mismatching of soil-N uptake patterns between fixing and reference plants to a background of rapidly changing ^{15}N enrichment plots and (2) reference plants do not consistently provide accurate % Ndfa estimates during legume development.

The %Ndfa estimate with ^{15}NNAM for individual sampling correlated only weakly with the estimates using ^{15}NEM (Figure 4.19). Even comparing the %Ndfa of Gliricidia in only Jun-03, when both methods did not differ significantly, the correlation between both methods was not significant (R<0.1).

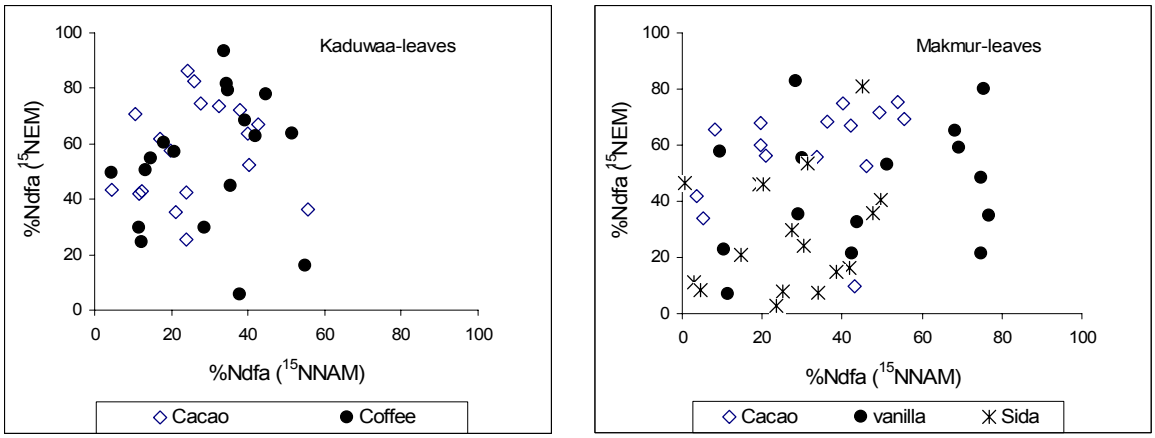

Figure 4.19: Relationship of proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia determined ^{15}N enrichment method (^{15}NEM) and ^{15}N natural abundance method (^{15}NNAM) in Kaduwaa and Makmur (n=5, four times of sampling, all sampling points)

Similar %Ndfa estimates using both approaches have been obtained under a variety of field conditions (Bremer and van Kessel 1990; Androsoff et al. 1995; Stevenson et al. 1995; Peoples et al. 1996; Hariah et al. 2000). However, a poor correlation between individual estimates from the two approaches was also observed (Androsoff et al. 1995; Stevenson et al. 1995), suggesting that both methods may not be comparable. However, Bremer and van Kessel (1990) found a good agreement of both methods in 18 out of 21 comparisons with *Pisum sativum* and *Lens culinaris* as fixing crops and *Tritucum aestivum*, *Linum usitatissimum, Hordeum vulgare*, *Brassica napus* as reference crops. Handley and Scrimgeour (1997) criticized the comparability of both

methods, as a good agreement could only be found in the mean estimates, but a correlation of individual estimates (paired-sampling) was lacking. They concluded that both methods measure different processes. On the other hand, Boddey et al. (2000) and Walley et al. (2001) stated that the generally observed poor agreement between both methods may be caused by high spatial variability in the controlling environmental variables, and that the two approaches basically measure the same process.

The %Ndfa of Gliricidia using atom% ^{15}N excess and $\delta^{15}N$ value in the twigs determined with ^{15}NEM and ^{15}NNAM were not significantly affected by the use of cacao, coffee and sida as reference plants. However, the comparison was only done for Oct-02 (12 weeks after the enrichment of ^{15}N fertilizer, Table 4.11). Uncertainty and some errors can occur when using the twigs to calculate the %Ndfa of fixing trees. The results in this study show that there was a higher variability in the individual %Ndfa estimates using all reference plants. Using the ^{15}NNAM, in some cases a negative estimate was obtained with cacao and coffee as the reference plants. Furthermore, it was difficult to visually determine whether the twigs of both fixing and reference plants were of the same age and in the same stage of development, which could have led to over- or underestimation of the %Ndfa of the fixing plant.

Table 4.11. Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia determined with ^{15}N enrichment method (^{15}NEM) and ^{15}N natural abundance method (^{15}NNAM) using atom% ^{15}N excess and $\delta^{15}N$ value in twigs in Oct-02 in Kaduwaa and Makmur

Site	Non-fixing Reference plant	%Ndfa		Paired *t*-Test Probability
		^{15}NEM	^{15}NNAM	
Kaduwaa	Cacao	52.2	36.6	ns
	Coffee	58.1	39.5	ns
Makmur	Cacao	70.6	48.8	ns
	Sida	58.6	43.7	ns

ns = not significant; n= 5

The %Ndfa of Gliricidia using the litter with both methods with cacao and coffee as reference plants fluctuated and did not show a uniform trend for plant and time of sampling (Table 4.12). In Kaduwaa, however, the %Ndfa of Gliricidia was not affected with cacao as the reference plant, and the individual %Ndfa shows higher variability. In some cases, with cacao (Oct-02) and coffee (Oct-02 and Mar-03), the %Ndfa of Gliricidia with ^{15}NNAM was less than 10 %. The difficulty was to have the

same stage of development of the litterfall of both Gliricidia and the reference plants, which is very different with respect to plant physiological processes such as mobility of N in the leaves (from old tissue to young tissue) before the leaves fall. Other difficulty was to make sure that the litterfall was exactly from the targeted tree (though using litter trap, there was some possibility that the litter mixed with the litter from non target tress). Therefore, using the litter was not recommended in estimating the %Ndfa of fixing trees, thought these data are crucial to find out the recycling of N from the fixing and reference trees to the system.

Table 4.12: Proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia determined with ^{15}N enrichment method (^{15}NEM) and ^{15}N natural abundance method (^{15}NNAM) using atom% ^{15}N excess and $\delta^{15}N$ value in litter at different times of sampling in Kaduwaa and Makmur

Site	Non-fixing Reference plant	Times of sampling	%Ndfa		Paired *t*-Test Probability
			^{15}NEM	^{15}NNAM	
Kaduwaa	Cacao	Oct-02	55.7	2.2	$P < 0.01$
		Dec-02	30.9	63.6	$P < 0.05$
		Mar-03	31.7	45.6	ns
		Jun-03	31.7	12.0	$P < 0.05$
		Mean	40.8	30.9	ns
	Coffee	Oct-02	67.7	2.5	$P < 0.01$
		Dec-02	54.3	48.4	ns
		Mar-03	55.2	8.9	$P < 0.05$
		Jun-03	70.2	31.2	$P < 0.01$
		Mean	54.3	22.8	$P < 0.01$
Makmur	Cacao	Oct-02	94.4	14.4	$P < 0.01$
		Dec-02	78.2	34.0	ns
		Mar-03	86.9	64.1	$P < 0.01$
		Jun-03	74.7	15.0	$P < 0.01$
		Mean	83.0	32.8	$P < 0.01$

ns = not significant; n= 5

4.4 Infection potential

Mean nodule fresh weight, number of nodules and infection potential were significantly higher in the soil at Kaduwaa than in the soil at Makmur (P<0.01; Figure 4.20). This was the case for both indicator plants, *Vigna unguiculata* and *Vigna radiata*. In the Kaduwaa soil, 165±33 nodules plant^{-1} were found, nodule fresh weight amounting to 76±12 g plant^{-1}, and the infection potential due to Rhizobium in the soils reached 5.5±1.1 nodules 100 g soil^{-1}, all values being almost four times higher than those

measured in Makmur. This was lower than the 8.2 nodules 100 g soil^{-1} observed with *Vigna unguic*ulata as indicator plant by Thilen-Klinge (1997) in young secondary vegetation in Brazil. She also reported that the nodulation on the roots of tree legumes was found up to 40 cm depth.

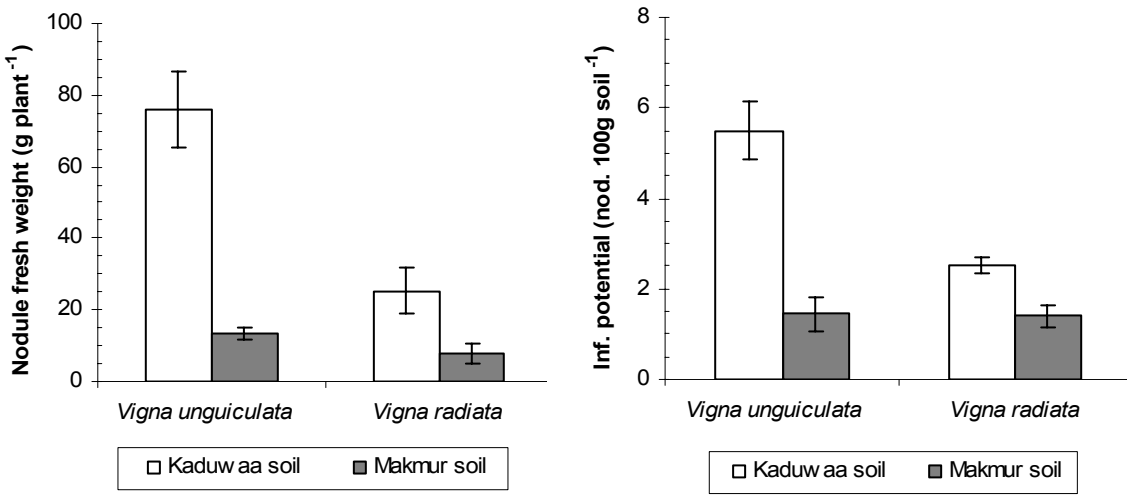

Figure 4.20: Mean nodule fresh weight and infection potential of soil using *Vigna unguiculata* and *Vigna radiata* as indicator plants in Kaduwaa and Makmur; n=3, bars represent standard error of the means

4.5 Leaf litterfall and pruning of Gliricidia

Total dry matter (DM) of the leave litterfall of Gliricidia was higher in Makmur (31.5 g m^{-2} 28-days^{-1}) than in Kaduwaa (24.7 g m^{-2} 28-days^{-1}; $P< 0.01$). However, N accumulation of leaf litterfall of Gliricidia did not differ significantly at both sites (Appendix 20), which was due to the lower N concentration in the leaf litterfall in Makmur (1.75 %) than in Kaduwaa (2.20 %). The dry matter and N accumulation of leaf litterfall at both sites fluctuated depending upon the time of sampling (Figure 4.21 and 4.22). In Makmur, the significantly highest leaf litterfall was observed from 12 December to 8 January and from 3 March to 3 April (28 days). In Kaduwaa this was the case from 19 September to 15 October 2002.

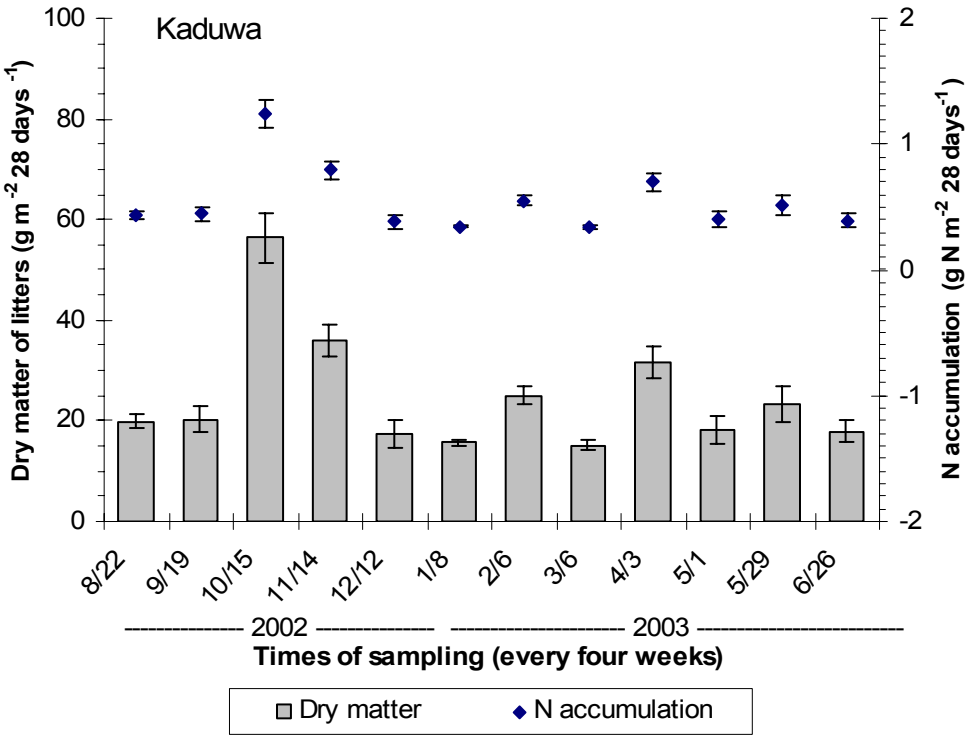

Figure 4.21: Dry matter and N accumulation of Gliricidia leaf litterfall (4-week intervals) in Kaduwaa; n=5; bars represent standard error of the means

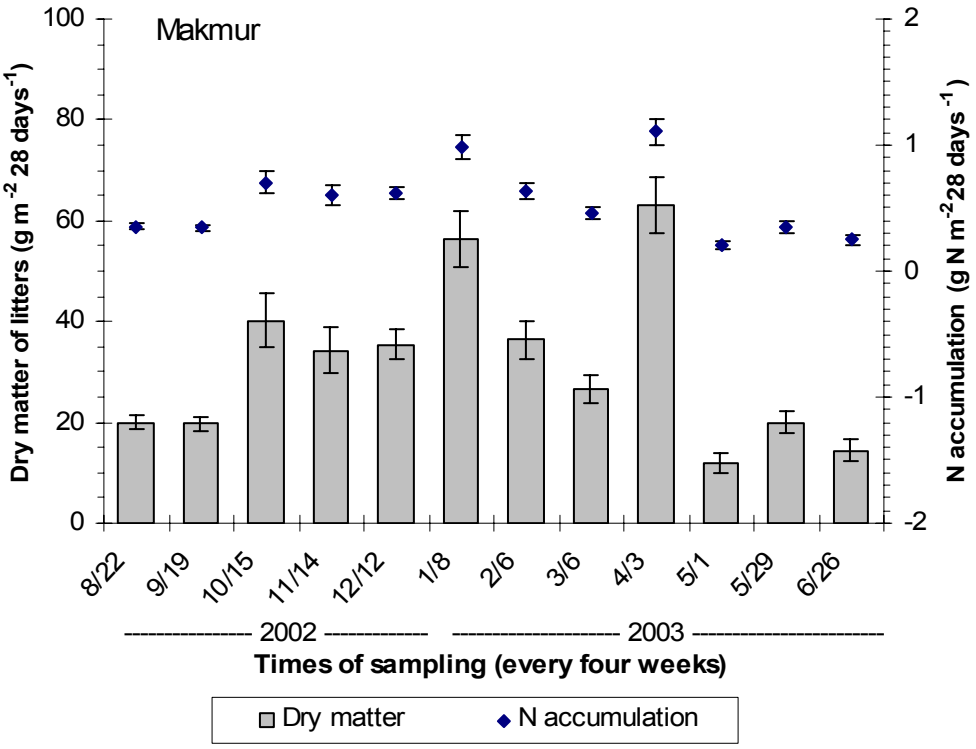

Figure 4.22: Dry matter and N accumulation of Gliricidia leaf litterfall (4-week intervals) in Makmur; n=5; bars represent standard error of the means

Total N input of Gliricidia via litterfall during 48 weeks of the study period was 68.4 kg N ha^{-1} in Kaduwaa and 64.8 kg N ha^{-1} in Makmur. This is higher than reported by other researchers. For example, in Northern Queensland, Australia, leaf litterfall of monoculture Gliricidia (0.5 m x 1.5 m spacing) at 104-117 weeks after planting was equal to 0.99 g N $plant^{-1}$ amounting to 52.8 kg N ha^{-1} yr^{-1}, which is equal to 6 % of the fixed N of the whole plant (Peoples et al. 1996). In Prise d'Eau, Guadeloupe, total accumulated biomass of Gliricidia litterfall in unpruned plots 17 weeks after regrowth was 50 kg DM ha^{-1} or 12.8 kg N ha^{-1} (i.e. 38.4 kg N ha^{-1} yr^{-1}; Nygren and Cruz 1998). The difference between these values and those in this study may be due to the age of the Gliricidia plants, i.e., 2-3 years as compared to 7-8 years in this study, and the density of the plants and litter N concentration.

In Kaduwaa, Gliricidia was only pruned once at the end of Oct-02 (start of rainy season), while in Makmur, Gliricidia was pruned twice, i.e., at the end of Oct-02 and mid Apr-03. The N accumulation of Gliricidia leaf pruning in Kaduwaa was slightly higher than in Makmur. In Kaduwaa, total Gliricidia leaf pruning (sampling from 12 m x 12 m with five replications) was 477 kg DM ha^{-1}, which was equal to 18.9±2.9 kg N ha^{-1}. In Makmur, the first and second leaf pruning resulted in 204 and 317 kg DM ha^{-1} equal to 7.1±1.7 and 10.9±2.5 kg N ha^{-1}, respectively. Total of both leaf prunings was 17.9±1.7 kg N ha^{-1}.

4.6 Total aboveground biomass and N content of Gliricidia

The linear regression analysis (Appendix 21 and 22) of the ln-transformed aboveground biomass as well as N content with ln-transformed basal diameter of Gliricidia is presented in Figure 4.23. The proportion of variation accounted for by the models (R^2) was 0.96 and 0.93 for aboveground biomass and N content, respectively. Independent of these linear regressions, the results also show that the N concentration declined with increasing basal diameter (e.g., from 1.35 % at 5 cm basal diameter to 1.15 % at 16 cm basal diameter; Appendix 22). This is in accordance with the general observation that, with increasing basal diameter, woody parts with a relatively lower N-content prevail.

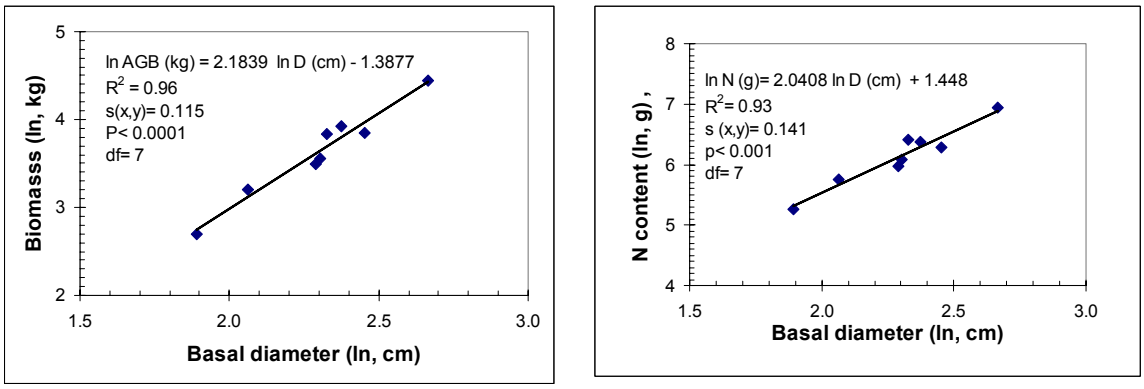

Figure 4.23: Linear regression of aboveground biomass and N content of Gliricidia with basal diameters; ABG= aboveground biomass, D= basal dimater

Total population of Gliricidia was 569 and 572 trees ha^{-1} in Kaduwaa and Makmur, respectively. Though in Makmur the spacing of Gliricidia (4 m x 4 m) was wider than in Kaduwaa (3 m x 4 m), the total population of Gliricidia was almost the same at both sites due to the death and the thinning of Gliricidia in Kaduwaa to provide more sun-light to the system. Combining tree numbers and the regression analysis, the total aboveground biomass of Gliricidia amounted to 26.6 Mg DM ha^{-1} in Kaduwaa and 30.6 Mg DM ha^{-1} in Makmur. Total above ground N content of Gliricidia was 321 kg ha^{-1} in Kaduwaa and 369 kg ha^{-1}, and thus slightly higher, in Makmur. The higher aboveground biomass and N content in Makmur may mainly be caused by differences in the age of Gliricidia (8 and 8.5 years in Kaduwaa and Makmur, respectively) and management practices by farmers (such as pruning and cutting) at both sites. Taking into account that the plants at both sites were around 8 and 8.5 years old meant that Gliricidia had accumulated on average around 40 and 43 kg N ha^{-1} yr^{-1} in Kaduwaa and Makmur, respectively.

4.7 Nitrogen balance in cacao agroforestry system

Two factors regulate the amount of N_2 fixed by legumes, i.e., the amount of N accumulated during growth, and the proportion of N that is derived from symbiotic N_2 fixation (Peoples et al. 1997). Assuming that the %Ndfa of Gliricidia determined with ^{15}NNAM (31-34 %Ndfa) and ^{15}NEM (53-57 %Ndfa) using cacao and coffee as reference plants are valid, the BNF in the cacao agroforestry system contributed around 13-22 kg N ha^{-1} yr^{-1} as the stock in the Gliricidia trees and 28-47 kg N ha^{-1} yr^{-1} as the recycled residue into the soil.

In Kaduwaa, the exported harvest product as reported by the farmers was around 1050 kg DM ha^{-1} yr^{-1} of cacao beans and 480 kg DM ha^{-1} yr^{-1} of coffee beans, and in Makmur around 1250 kg DM ha^{-1} yr^{-1} of cacao beans. No information could be obtained about the harvested amounts of vanilla. In Kaduwaa, N withdrawal by the harvested products equaled 39.5 kg N ha^{-1} yr^{-1} (29.9 and 9.6 kg N ha^{-1} yr^{-1} for cacao and coffee beans, respectively). In Makmur, this was 35.2 kg N ha^{-1} yr^{-1} (only cacao beans). In a nearby comparable agroforestry site, slightly higher N withdrawals in the harvested products (57 kg N ha^{-1} yr^{-1}) were observed than from the study site (Dechert 2003). Higher N losses in this agroforestry system were due to differences in the plant composition and management practices (plant spacing); 540, 1140, and 900 kg DM ha^{-1} yr^{-1} cacao, coffee and candle nut, respectively, were harvested.

Assuming there is no N-change in the soil and that all wood is exported, and considering the remaining fluxes of N into and out of the system as reported by Dechert (2003) as well neglecting N losses through surface run-off and volatilization (assumed to be insignificant), the N balance in the system ranged from -15 to +17 kg N ha^{-1} yr^{-1} (Figure 4.24). Hairiah et al. (2000) calculated the N balance of Gliricidia in a hedgerow intercrop system in Indonesia. They observed that BNF input from Gliricidia in the system was sufficient to sustain crop yields and associated N withdrawals. The amount of N_2 fixed (35-38 kg N ha yr^{-1}) by Gliricidia was sufficient to compensate for the whole system N off-take of approximately 34-37 kg N ha yr^{-1} during the first two years. A positive N balance was also detected in a cacao agroforestry system in Costa Rica (Escalante et al. 1984 *in* Beer 1988; Roskoski and van Kessel. 1985). The NFTs such as *Erythrina* or *Inga* spp. were able to annually fix 35-60 kg N ha^{-1}. This was sufficient to replace the N exported in the crop harvest from plantations where no or little organic fertilizer was applied. Further advantages of NFTs have also been observed. Planted as a shade tree in cacao plantations in the humid tropics, *E. poeppigiana* conserved soil and contributed to high and sustainable cacao yields (Beer et al. 1998). It has also been reported that decomposed litter of Gliricidia increases nutritional status, water holding capacity and bulk density of the soil (Rosecrance et al. 1992; Arachchi and Liyanage 1998). According to Nygren et al. (2000), N release from litterfall of Gliricidia trees may reach 20 kg N ha^{-1} yr^{-1}.

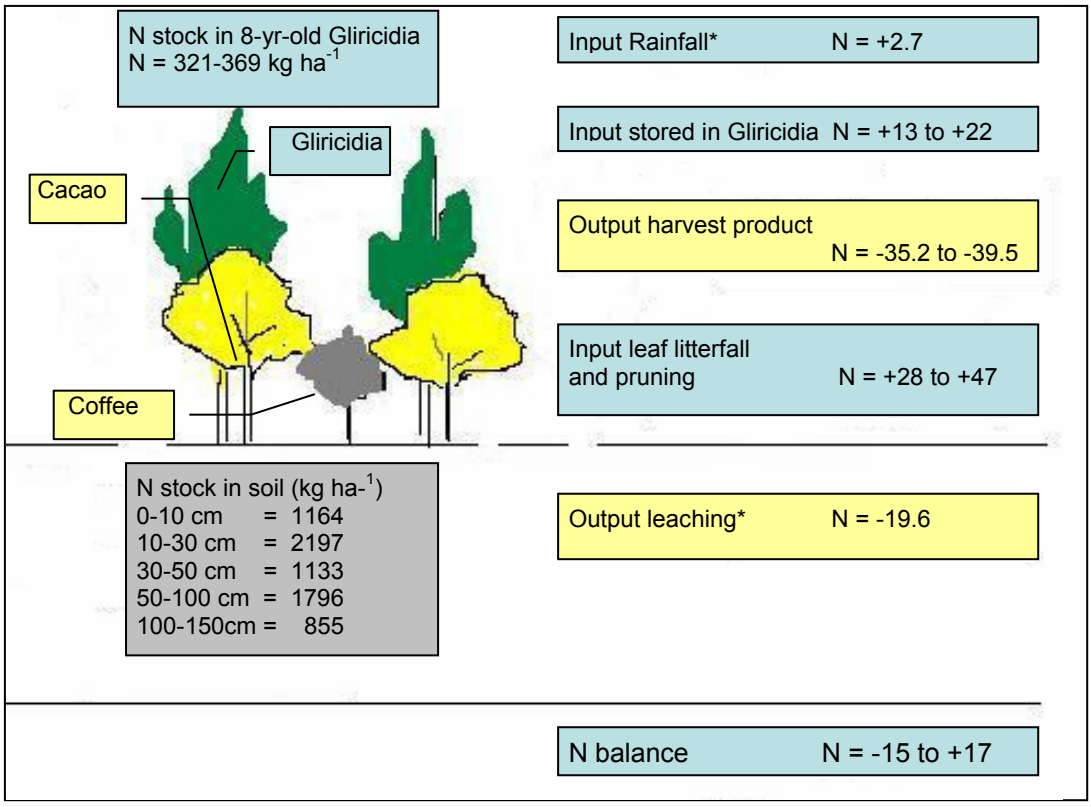

Figure 4.24: Contribution of biological nitrogen fixation to N balance in cacao agroforestry system; unless otherwise stated, all results in kg N ha^{-1} yr^{-1}; *based on Dechert 2003.

5 GENERAL DISCUSSION AND CONCLUSIONS

5.1 Quantifying %Ndfa of Gliricidia

Nitrogen fixing trees play a major role in improving soil N fertility. However, there are only a few studies available evaluating their capacity to fix N_2 especially under field condition due to methodological difficulties. Comparative evaluation of BNF in the cacao agroforestry system in Central Sulawesi, Indonesia, using ^{15}N methodologies (^{15}NEM and $^{15}NNAM$) revealed that both methods vary in the estimation of the %Ndfa of Gliricidia. There is no agreement between both methods based on mean and on individual paired sampling. Though in this study the $^{15}NNAM$ seems more reliable than the ^{15}NEM, it is difficult to conclude that in this system the former provides more accurate estimates than the latter. Both methods have distinct limitations. However, with careful use, both techniques should provide valuable information.

Furthermore, large variations in the %Ndfa of Gliricidia are based on the site conditions. These are linked to climate, soil chemical characteristics, and the potential infection by Rhizobium in the soil. Seasonal variation in the %Ndfa of Gliricidia were observed in this study at both sites at the onset of precipitation. Dry conditions resulted in lower %Ndfa values. However, high precipitation does not automatically result in high %Ndfa values. Lower N_2 fixing activity due to lower precipitation was also reported by Bremer and van Kessel (1990). Sprent (1972) observed that nodule activity is reduced in response to dry and water-logged conditions, and that maximum N_2 fixation occurred when soils were near field capacity. Low available P and pH in the study sites are suspected to be responsible for the low %Ndfa. Finally, the lower infection potential by Rhizobium in the soil in Makmur than in Kaduwaa could also the reason for the lower %Ndfa in the former site.

5.1.1 Nitrogen-15 enrichment method

The atom% ^{15}N excess increases significantly above the abundance level not only in each respective soil depth (Figure 4.1), but also in the fixing and reference plants (Table 4.3), at first sight satisfying the above-stated needs for the accurate estimation of %Ndfa of Gliricidia. However, the atom% ^{15}N excess in the soil (0-30 cm depths) declines rapidly; only 28 days after the enrichment it had almost returned to the natural abundance level (Figure 4.2). This may be a source of error if the fixing and reference

plants have different patterns of uptake of plant-available soil ^{15}N. For example, if the fixing plant absorbs most of the plant-available soil ^{15}N earlier after ^{15}N application than the reference plant, the %Ndfa would be underestimated. Analogously, the reverse situation would lead to an overestimate. The fact that the %Ndfa estimate varies among the reference plants indicates that these plants showed different ^{15}N-uptake patterns. Ledgard et al. (1984) and Witty (1983) also observed different %Ndfa estimates determined with the ^{15}NEM using different reference plants. They attributed this to the change in the ^{15}N enrichment of plant-available soil N with time interacting with differences in the pattern of N assimilation between the fixing and reference plants.

The application of ^{15}N fertilizer through injection successfully distributes the ^{15}N fertilizer vertically down the soil profile, but not laterally. This may lead to errors in the %Ndfa estimate, since the basic assumption that the ^{15}N fertilizer is well distributed laterally is not met. This basically provokes the same consequences as stated above if the roots of the plants do not have the same spatial (above all lateral) pattern to absorb the plant-available soil ^{15}N. This might be the case for sida and vanilla, which are much smaller than coffee and cacoa, and thus possibly do not have a rooting system that can access and take up labeled soil as effectively as coffee and cacao. The lower estimates of %Ndfa of Gliricidia using these plants compared to cacao and coffee as reference plants support this assumption.

The temporal pattern of the isotopic composition of the plant-available soil ^{15}N was greatly affected by the timing of the ^{15}N fertilizer application (Figure 4.4). Increasing atom% ^{15}N excess detected both in the soil and in the plant (plant-available soil ^{15}N) did not increase the %Ndfa value and the accuracy of the estimate. Pooled data of the %Ndfa Gliricidia over all reference plants show that in Kaduwa the mean estimates and standard errors of Ndfa were 57±10, 44±5, 44±6, and 40±12 in Oct-02, Dec-02, Mar-03 and Jun-03, respectively, and in Makmur 51±6, 46±7, 44±11, and 36±6 in Oct-02, Dec-02, Mar-03 and Jun-03, respectively. This is not in agreement with the results of Peoples et al. (2001), who reported that plant-available soil ^{15}N influenced the accuracy of the %Ndfa estimate, i.e., the higher the plant-available soil ^{15}N, the greater the potential accuracy of subsequent %Ndfa estimates. The results in this study confirm the findings of Peoples et al. (1989) that an application of, for example, less than 5 kg N ha^{-1} of ^{15}N-labeled is preferable and does not affect N_2 fixation. However, with the rapid

decline of atom% ^{15}N excess in the soil, splitting the applications to four times or at least more than two times, or applying less N fertilizer with higher ^{15}N enrichment (e.g., 99 atom% ^{15}N; Gathumbi et al. 2003) could increase the atom% ^{15}N excess detected in the soil and plant compartments, and hence, result in a more accurate %Ndfa estimate of fixing trees. This would be valuable when dealing with reference plants that have different rooting patterns.

The atom% $^{15}NH_4^+$ and $^{15}NO_3^-$ excess in the soil at the end of the experiment (Jun-03) was not affected by soil depth (Table 4.1). On the contrary, the NH_4^+ and NO_3^- concentrations in the soil decreased significantly with depth (Table 4.2) suggesting that based on the soil conditions in this study, and when the ^{15}N fertilizer was injected to the soil in order to increase the plant-available soil ^{15}N in deeper soil layers, double-labeled $^{15}NH_4^+$-$^{15}NO_3^-$ is not more crucial than single-labeled $^{15}NH_4^+$ or $^{15}NO_3^-$.

Serious errors can occur when the ^{15}NEM is applied in combination with unsuitable reference plants (Fried et al. 1983; Witty 1983). Hence, the right choice of reference plants in ^{15}NEM studies is essential. The major concern is whether fixing and reference plants, which may differ both morphologically and physiologically, obtain the same portion of labeled and non-labeled ^{15}N from the soil. In this study, the %Ndfa of Gliricidia varied significantly among the reference plants used. The individual sampling times and differences in the rooting patterns of the reference plants might have been the reasons for these differences (Figure 4.6 and 4.7). The roots of vanilla and sida prevail in the 0-30 cm depth. Hence, the rapid decline of the atom% ^{15}N excess in the top soil layer reduces the ^{15}N-absorbed by sida and vanilla and accordingly reduces the %Ndfa of Gliricidia. On the other hand, cacao and coffee have deeper roots and are therefore able to absorb plant-available soil ^{15}N in deeper depths, thus increasing the %Ndfa of Gliricidia. Several researchers suggested that the root system of fixing and reference plants may be allowed to differ both in size and structure, as long as most of the plant-available soil N and labeled ^{15}N fertilizer are accessible to both. They furthermore argued that the ^{15}NEM is not distorted by reference plants that explore different soil volumes, since the majority of the ^{15}N label and soil N are found in the upper horizons (Fried et al. 1983; Peoples et al. 1989; Danso et al. 1992). However, these reports do not coincide with the finding of this study, but may lead to errors in the % Ndfa estimate for Gliricidia in the cacao agroforestry system. For that reason, cacao and coffee may be

better reference plants than vanilla and sida when the ^{15}NEM is applied in cacao agroforestry systems.

The %Ndfa of Gliricidia varied considerably with different plant parts. The main concern was the inconsistency of the %Ndfa estimate using atom% ^{15}N excess in the twigs and in the litter even with the same reference plant. The atom ^{15}N excess in the twigs may also contain unlabelled N already existing prior to the labeling, suggesting that differences between fixing and reference plants may lead to an under- or overestimation of the %Ndfa. Shearer and Kohl (1988) state that the ^{15}N content in the plant at the end of an experiment reflects not only the ^{15}N content of the labeled soil N pool, but also the amount of unlabelled ^{15}N present in the plant at the beginning of the experiment. In addition, remobilization of N from old to young tissue, mineralization of litter-N, and different physiological process between plants may increase inaccuracy, implying that the %Ndfa estimate of the fixing plant using atom% ^{15}N excess in the litter may not be accurate. Therefore, in this study, using the tissue of young leaves may be more appropriate than using twigs and litter assuming that it may not contain N assimilated prior to the beginning of the experiment. Peoples et al. (2001) state that sampling the whole tree is not possible, and that it might more satisfactory to sample leaves or other newly grown plant parts.

5.1.2 Nitrogen-15 natural abundance method

A number of basic requirements have to be met for accurate estimates of %Ndfa using the ^{15}NNAM (Shearer and Kohl 1986; Peoples et al. 1989; Ladha et al. 1993): These are: 1) The δ^{15}N value of plant-available soil N in the soil should be preferably > 5 ‰; 2) the natural enrichment of plant-available soil N should be uniform across the experimental study sites and not rapidly change with depth or time; 3) appropriate reference plant have to provide a measure of the δ^{15}N value of plant-available soil N; and 4) the whole plant ^{15}N composition has to be represented by the plant part used for analyses.

It is generally recommended that the δ^{15}N value of plant-available soil N should be higher than 6 ‰, as the accuracy of the ^{15}NNAM decreases at lower natural enrichments (Shearer and Kohl 1986; Ledgard and Peoples 1988). For use in tree-based fallow systems, Gathumbi et al. (2002) suggest that it should preferably be higher than 5

‰. In this study, the results show that the $\delta^{15}N$ value of plant-available soil N depends on the reference plant used and the site (Figure 4.12). However, the %Ndfa of Gliricidia is also influenced by the $\delta^{15}N$ value of the fixing plant. Though the $\delta^{15}N$ value of plant-available soil N in Makmur was less than that in Kaduwaa, the %Ndfa of Gliricidia did not differ significantly with cacao as the reference plant, since in Makmur the $\delta^{15}N$ value of Gliricidia is also less than in Kaduwaa. Uncovich et al. (1994) stated that a difference in the $\delta^{15}N$ value of plant-available soil N and atmospheric N_2 as low as 2 ‰ can be used in pastures using carefully matched reference plants and paired sampling procedure. Such a procedure was used in this study to provide for a close match of external factors. This suggests that the level of the $\delta^{15}N$ value of plant-available soil N at both sites is sufficient for ^{15}NNAM studies.

One of the major advantages of the ^{15}NNAM over the ^{15}NEM is the use of natural enrichment of the soil N pool, which has smaller variations with soil depth, thus providing a good probability for fixing and reference plants to explore a soil with identical $\delta^{15}N$ composition. Hence, the choice of the reference plant would be less crucial. In this study, however, this small variation of the $\delta^{15}N$ value of total soil N increases significantly with depth (Figure 4.10). In addition, in only three out of five times of sampling were the $\delta^{15}N$ values of total soil N not affected by depth This means that the hypothesis that the selection of the reference plant is less crucial with the ^{15}NNAM may not be held. Thus, the uniformity of the $\delta^{15}N$ value of plant-available soil N with depth is more important than the $\delta^{15}N$ value of total soil N. Researchers in Australia, the Philippines, Indonesia and Kenya reported that the $\delta^{15}N$ value of total soil N increased with depth, while the $\delta^{15}N$ value of plant-available soil N did not (Ledgard et al. 1984; Ladha et al. 1993; Cadisch et al. 2000; Gathumbi et al. 2002). In the present study, this was only the case for plant-available soil N in Makmur but not in Kaduwaa (Table 4.5). In the latter case (plant-available soil N in Kaduwaa), this may have resulted in errors in the %Ndfa of Gliricidia due to a mismatch of rooting patterns and uptake of plant-available soil N between fixing and reference plants. Rooting depth is suggested as one factor affecting the $\delta^{15}N$ value among reference plants, since deep-rooting plants may reach soil N-enriched in ^{15}N, while shallow-rooted plants may reach soil N slightly depleted in ^{15}N (Virginia et al. 1989).

Nevertheless, it cannot be concluded that the %Ndfa of Gliricidia will not differ among reference plants even with similar $\delta^{15}N$ values of depth-dependent plant-available soil N. As in Makmur, the %Ndfa of Gliricidia differed among the reference plants (cacao, vanilla and sida), suggesting that the right choice of reference plant is crucial even with similar $\delta^{15}N$ values of depth-dependent plant-available soil N. This assumption is also supported by the results at the end of the experiment (Jun-03), which show that though the $\delta^{15}N$ value of plant-available soil $^{15}NH_4^+$ and $^{15}NO_3^-$ was not significantly different with depth (Table 4.6), the %Ndfa estimate varied considerably with different reference plants.

Another basic requirement of the ^{15}NNAM is that the fixing and reference plants explore a soil N pool of identical $\delta^{15}N$ values of time-dependent plant-available soil N. In this study, the $\delta^{15}N$ values of plant-available soil N as assessed for the reference plants and the $\delta^{15}N$ values of the fixing plant varied considerably with time (Figure 4.12). These variations may be affected by the total precipitation during sampling time, which, therefore, affect the %Ndfa of Gliricidia. At the end of the rainy season, the %Ndfa of Gliricidia ranged between 50 and 68 %. After two months of less rain, values declined sharply, ranging between 13 and 38 %. A rapid change of plant-available soil N with time was observed by Ladha et al. (1993) in alley cropping systems in the Philippines and Turner et al. (1987) in Australia. In this situation, errors can occur in the calculation of BNF if the fixing and reference plants exhibit different rooting depths and/or have different N uptake characteristics with time (Boodey et al. 2000). Shearer and Kohl (1988) suggest that although no differences in the $\delta^{15}N$ value could be related to rooting depth, when selecting the reference plants it is especially important to choose plants that feed from similar soil volumes and show the same temporal pattern of N uptake. Therefore, the choice of appropriate reference plants under these conditions is critical.

The choice of appropriate reference plants is, however, extremely difficult to determine in an experimental way. Though cacao, coffee, and sida resulted in similar trends of the $\delta^{15}N$ value of plant-available soil N over time, the %Ndfa of Gliricidia varied depending on the reference plant used. In addition, the trend of $\%N_{tot}$ in the leaves of reference plants also varied considerably compared to that in the fixing tree making the appropriate choice of a reference plant difficult. Given this, it appears to be

plausible to use the average %Ndfa estimate obtained from all reference plants as recommended by Boddey et al. (1995). In addition, Boddey et al. (2000) stated that the inconsistency of the $\delta^{15}N$ values of reference plants over time is a further evidence that the %Ndfa estimate should not rely solely on one so-called 'appropriate' reference plant. Consequently, Shearer and Kohl (1988) postulate that due to the variation in the $\delta^{15}N$ value among reference plants, the ^{15}NNAM is a semi-quantitative approach.

The %Ndfa of Gliricidia varied considerably with different plant parts. In general, the %Ndfa of Gliricidia based on the $\delta^{15}N$ value in the twigs resulted in higher estimates than that based on leaves and litter. The main problem was related to the inconsistency of the %Ndfa estimate using twigs and litter even with the same reference plant. Using the $\delta^{15}N$ value in the leaves resulted in less variation in the %Ndfa estimate than when using that of twigs and litter. Thilen-Klinge (1997) in comparing the $\delta^{15}N$ value of young leaves with old leaves suggests that using young leaves gives more consistent %Ndfa values, since they show less variability. Furthermore, even in the same tissue, the $\delta^{15}N$ value often changes during growth and development (Boddey et al. 2000). Shearer and Kohl (1988) state that the ^{15}N content of the plant at the end of experiment reflects not only the ^{15}N content of the soil N pool, but also the amount of ^{15}N present in the plant at the beginning of the experiment. In addition, re-allocation of N from old tissue to young tissue before the senescence and mineralization of litter N contributes to inaccuracies in the %Ndfa of the fixing plant. Therefore, leaf tissue is more appropriate than twigs and litter. As already stated by Peoples et al. (2001), sampling whole trees is basically not possible, or at least very inconvenient. The satisfactory alternative could be to sub-sample leaves and re-grown plant parts. Furthermore, the difference between their $\delta^{15}N$ value and that of other components is not large (Boddey et al. 2000).

5.1.3 Comparison of ^{15}NEM and ^{15}NNAM

The %Ndfa of Gliricidia with cacao and coffee as reference plants determined with ^{15}NEM were 23-31 % higher than with ^{15}NNAM. Hairiah et al. (2000) also could not match both methods to determine the %Ndfa of Gliricidia. The %Ndfa estimates using ^{15}NEM exceeded those of ^{15}NNAM by 18 %. Bergersen and Turner (1983) came up with a mean difference (four separate measurements) between both methods of 13 %;

the maximum difference was even 33 %. Assuming that both ^{15}NEM and ^{15}NNAM are valid methods to determine leguminous N_2 fixation (Shearer and Kohl. 1986), the %Ndfa estimates of both methods should be comparable, based on both mean and individual estimates. In theory, if the labeling procedures results in an 'operationally' uniform distribution or, alternatively, if any inconsistencies are expressed equally in the fixing and reference plants, no difference in %Ndfa can be expected (Rennie 1986).

The results in this study show that vertical and temporal variations of ^{15}N in the soil with the ^{15}NNAM are smaller than with the ^{15}NEM. Less variability of natural abundance of ^{15}N in the soil did not result in similar estimates of % Ndfa over time with different reference plants or sampling times. Thus, the occurrence of an isotope fractionation specific to the reference plant during N uptake may result in different estimates of %Ndfa for Gliricidia. Though the natural abundance of ^{15}N in the soil varies less, this does not automatically result in a lower susceptibility to errors associated with rooting depth of fixing and reference plants in the ^{15}NNAM than in the ^{15}NEM (Shearer and Kohl 1986). Stevenson et al. (1995) state that ^{15}NNAM and ^{15}NEM might even require different reference plants as the requirements for the reference plant differ between them. For example, the intensity of ^{15}N isotopic fractionation occurring during N-uptake by the plants has little effect on the %Ndfa estimated with the ^{15}NEM, but can strongly effect that estimated with the ^{15}NNAM (Shearer and Kohl 1986; Bremer et al. 1993). Therefore, the same precautions for choosing the reference plant should be taken when applying both the ^{15}NNAM and the ^{15}NEM (Witty 1983).

Choosing the most appropriate reference plants is likely to be more important than the problem of the non-uniform mixing of the applied ^{15}N, because any inherent error exists equally for the fixing and reference plants (Rennie 1986). The use of inappropriate a reference plant can lead to high variability in the estimates of %Ndfa. The disadvantage of the ^{15}NEM is that following the application of ^{15}N fertilizer, the plant-available soil N pool becomes enriched with ^{15}N and, following this, the ^{15}N:^{14}N ratio of the available soil N pool declines rapidly (Witty 1983). The appropriateness of a reference plant may be less of a problem for the ^{15}NNAM than for the ^{15}NEM, at least with respect to the temporal pattern of uptake of plant-available soil N during the growing season (Shearer and Kohl 1988).

Regarding the individual %Ndfa estimates for Gliricidia determined with both methods (paired-samples), a good agreement was only found in Jun-03, but not in Oct-02, Dec-02 or Mar-03. Consequently, the results of both methods might also be time dependent. The results show that the ^{15}N fractionation was affected by soil moisture (rainy seasons vs. dry season) and resulted in higher variations in the %Ndfa of Gliricidia with ^{15}NNAM. On the other hand, when the ^{15}NEM was applied, higher variations in %Ndfa were also detected in response to the decline of the atom% ^{15}N excess in the soil. Therefore, many samplings during the growing season are necessary before conclusions can be drawn whether both methods match or not. The results of this study show that if the sampling is only done at the end of the experiment, a good agreement of the mean estimate between both methods is attained with all reference plants used. However, at the other three times of sampling both methods produced significantly different amounts of %Ndfa. This was already claimed by Peoples et al. (1996), who highlighted the need of repeated measurements during growth and development before drawing conclusions about the relative ability of fixing plants to fix N.

Using the twigs to calculate the %Ndfa of Gliricidia, both methods result in similar %Ndfa estimates with different reference plants. Unfortunately, sampling was done only once (Oct-02), since it was expected that the estimates using twigs would not differ. In addition, using the twigs or stems may result in over- or underestimation of the %Ndfa due to ^{15}N stored in the fixing and reference plants prior to the start of the experiment. The %Ndfa of Gliricidia using the litter fluctuated with both methods, not showing any trend between plants and times of sampling, suggesting that differences between species in the internal turnover of N may largely affect the %Ndfa estimate. Sub-samples of leaves provide the best options, since they may provide the same information on $\delta^{15}N$ or atom% ^{15}N excess uptake by fixing and reference plants. This is especially important as leaves present the largest single pool of aboveground N biomass in many agroforestry systems (Peoples et al. 2001).

Although a good agreement between the two methods is found, this does not automatically imply that both methods provide a correct estimate for N_2 fixation (Witty and Ritz 1984; Bremer and van Kessel 1990; Cadisch et al. 2000). In some cases, the ^{15}NNAM is more reliable than the ^{15}NEM because the $\delta^{15}N$ value of reference plants is

uniformly stable, and does not have the uncertainty of the ^{15}NEM associated with the doubt about the effectiveness of uniformly labeling the plant-available soil N (Ledgard et al. 1985; Hamilton et al. 1993). Hőgberg (1997) stated that although the ^{15}NEM is theoretically more precise, it is by no means clear that in complex field situations it provides more accurate %Ndfa estimates than the ^{15}NNAM.

5.2 Role of BNF in cacao agroforestry system in Central Sulawesi

In this study, the fact that the BNF input to the N balance ranged from only -15 to +17 kg ha^{-1} yr^{-1} (Figure 4.24) does not mean that the BNF does not play a major role in maintaining soil N fertility and sustaining the production in the system. The N balance in this study was based on the aboveground biomass input only. It should also be considered that decomposing dead roots of Gliricidia may further contribute N to the system. Rowe (1999) measured a Gliricidia root biomass of 3900 kg DM ha^{-1} in hedgerow systems, which is equivalent to approximately 70 kg N ha^{-1}. In addition, the nodule turnover of Gliricidia may reach another 20 kg N ha^{-1} yr^{-1} (Nygren and Cruz 1998). Therefore, this would lead to a positive balance in the cacao agroforestry system.

In Central Sulawesi, more and more farmers are converting their traditional cacao agroforestry systems to "full-sun" cacao monocultures. It has been proved that this practice leads to a short-term increase in cacao production, but in the long run it not only increases stress and reduces the period of productivity, but also increases the need for fertilizer, especially N, and pesticides (Beer et al. 1998; Siebert 2002). Assuming that farmers in the study area have converted their traditional cacao agroforestry system to cacao monoculture, the N output from harvested products and leaching (based on Deacher 2003) ranges from 54.8 to 59.1 kg N ha^{-1} yr^{-1}. This is equivalent to approximately 119-128 kg urea (46 % N) ha^{-1}. With a price around Euro (€) 0.3 kg^{-1} (1 € = 10000 IDR), to maintain the soil fertility at the current level farmers have to pay € 36-38 for urea ha^{-1}. This is almost a minimum salary for one month in the region. The additional input for pesticides must also be considered. Furthermore, reducing the period of productivity of cacao in the monoculture system means extra costs for land preparation and new plants.

An economic analysis of cacao production in Ghana revealed that the extra expenditure and work associated with clear-felling and growing unshaded cacao with large amounts of fertilizer could be justified only when the cacao production was at least 3360 kg ha^{-1} yr^{-1} (Cunningham 1963). Though there is no such report for cacao agroforestry systems in Central Sulawesi, transferring these results would basically mean any the farmer who wants to shift his traditional cacao farming system to a cacao monoculture plantation will have to increase yields 2-3 times. In addition, poor knowledge and resources of farmers to maintain these crops without shade will reduce yields and in the long run lead to the abandonment of the plantations (Beer 1987). Therefore, improving management practices in traditional cacao agroforestry systems is a better option than converting to cacao 'monoculture' plantation systems. This can be achieved with regular pruning not only of the NFTs but also of the main plants and by returning the wood, instead of using it for fences and new shade trees elsewhere. Hence, the competition between plants for sunlight will be reduced leading to improved growth, development and yield of the plant. Nutrient leaching, especially areas with high rainfall, will also be reduced by the recycling of N by the deep-rooting shade trees. Finally, in contrast to cacao monoculture plantation systems, which need more industrially manufactured N and pesticides, the use of BNF in the traditional cacao agroforestry system will reduce greenhouse gas emissions and water pollution and promote a more sustainable use of agricultural land.

6 CONCLUSIONS AND RESEARCH NEEDS

6.1 Conclusions

Estimates of the contribution of BNF by *Gliricidia sepium* in the cacao agroforestry system in Central Sulawesi Indonesia indicate that the %Ndfa varies between ^{15}NEM and ^{15}NNAM, depending on the sites and the method used. This corresponds to BNF inputs at system levels between 31-34 % (^{15}NNAM) and 53-57 % (^{15}NEM), and 13-22 kg N ha^{-1} yr^{-1} as the stock in the Gliricidia trees and 28-47 kg N ha^{-1} yr^{-1} as the residue recycled into the soil. Though the contribution of BNF to N the balance in the system ranges from only -15 to +17 kg N ha^{-1} yr^{-1}, BNF in Gliricidia without doubt plays a major role in maintaining soil fertility and sustaining production, especially in traditionally managed cacao system.

The large variation in %Ndfa, i.e., of total amount of N_2-fixed by Gliricidia, is linked to (1) site, (2) ^{15}N methodology, (3) choices of the reference plants and (4) representative sampling strategies. Soil-available P, pH, infection potential, precipitation, and management practices (pruning) affect the N_2-fixed activities by *Rhizobia* of Gliricidia. Considering the ^{15}N methodologies applied, variations are due to the changes in ^{15}N with time and depth. A low lateral distribution of mineral ^{15}N in the soil might add to variability when the ^{15}NEM is applied, while when using the ^{15}NNAM this is due to the variations of the δ^{15}N value of mineral soil N with time and depth. Finally, different root distribution and uptake patterns of ^{15}N between fixing and reference plants and the different plant parts used in calculating the %Ndfa might also contribute to different %Ndfa values for of Gliricidia.

It may be concluded that the ^{15}NNAM is more reliable in calculating the %Ndfa of Gliricidia in the system than the ^{15}NEM, since the δ^{15}N value of plant-available soil N is uniformly stable with time and depth, and does not have the uncertainty of the ^{15}NEM associated with the doubt about the effectiveness of uniformly labeling the plant-available soil N. Regarding the contribution of BNF to N balances, improving management practices in traditional cacao agroforestry systems is a better option than converting to cacao ‘monoculture’ plantation systems.

6.2 Research needs

The findings in this study reveal that ^{15}N methodologies resulted in uncertainties with respect to the accuracy of the %Ndfa estimates for Gliricidia in the cacao agroforestry system. Rapid decline of ^{15}N enrichment in the soil with time and depth, and its limited lateral movement coupled with different root distribution and uptake patterns between fixing and reference plants turned out to be the main concern when applying the ^{15}NEM. Therefore, instead of splitting the application two times and injecting to different depths at a distance of 1 m x 1 m, four or more applications of smaller doses with higher atom% ^{15}N excess (99 %) and injected at a shorter distance (25-50 cm) should increase the accuracy of the estimates. Meanwhile, when applying the $^{15}NNAM$, instead of depending on the plant-available ^{15}N (as measured in the reference plants), determining the mineral soil ^{15}N regularly between sampling would provide better information on the change of mineral soil ^{15}N with time and depth, resulting in more accurate estimates.

With regard to management practices in the cacao agroforestry systems, regular pruning of Gliricidia, cacao and coffee, and residue management are the best options for maintaining soil fertility and sustaining the production. However, research is still needed to evaluate the amount and time of pruning during a season in connection with the N_2 fixation activities of Gliricidia and the production of cacao and coffee. In addition, data concerning the management of residues such as leaf litterfall and twigs/stems of these plants and the cacao pods as well as investigation of the N input by these components are also needed for a full description of the N cycle in the system.

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8 APPENDICES

Appendix 1: Comparison of the ^{15}N natural abundance method (^{15}NNAM) and ^{15}N enrichment method (^{15}NEM) in the proportion of N derived from the atmospheric N_2 (%Ndfa) of legumes in different cropping systems

Type of experiment	Fixing plant	Reference plant	%Ndfa		Agreement	Ref.
			^{15}NNAM	^{15}NEM		
Field (landscape)	*Pisum sativum*	*Brassica napus*	13-97 (44)**	0-93 (50)	similar but no correlation	1
Field	*Arachis hypogaea*	*Arachis hypogaea**	21-53	33-46	good	2
		Zea mays	16-44	23-33		
Field (hedgerow)	*Flemingia congesta, Gliricidia sepium*	*Pelthophorum dasyrachis*	37(G) 24(F)	55(G) 32(F)	^{15}NEM over-estimate to 18%	3
Field	*Medicago sativa*	*Lolium rigidum*, *Phalaris aquatica*	85-86(L) 64-81(P)	50-70(L) 70-88(P)	similar	4
Field	*Calliandra calothyrsus, Gliricidia sepium*	*Senna spectabilis, Panicum maximum*	24-84 (59,C) 56-89 (72,G)	65 (C), 70 (G)	similar at most harvest	5
Field (landscape)	*Pisum sativum*	*Brassica napus*	71-84	70-92	similar, no correlation	6
Field (landscape)	*Cicer arietinum*	*Tritucum aestivum*	36-69.7 (54.6)		lack of strong correlation	7
Field	*Pisum sativum* , *Lens culinaris*	*Tritucum aestivum*, *Linum usitatissimum, Hordeum vulgare*, *Brassica napus*	35-81	36-80	good agreement 18 out of 21 comparison	8
Filed (Eucalyptus forest)	*Accacia melanoxylon, Accacia mucronata*	*Poa sieberiana*, Opportunistic ref. plant	25-40, 22-48	6-58, 29-67	only one out of three samplings	9

**Uninoculated legume; ** Value in brackets is the mean; (1) Androsoft et al. 1995, (2) Cadisch et al. (2000);(3) Hairiah et al. (2000); (4) Ledgard et al. (1985); (5) Peoples et al. 1996; (6) Stevenson et al. 1995; (7) Walley et al. (2001); (8) Bremer and van Kessel (1990); (9) Hamilton et al. (1993)*

Appendix 2: Effect of soil depth and time of sampling on atom% ^{15}N excess and $\%N_{tot}$ in soil in Kaduwaa and Makmur

Treatment		Kaduwaa		Makmur	
		Atom% ^{15}N excess(1)	N_{tot} (%)(2)	Atom% ^{15}N excess(1)	N_{tot} (%)(2)
	df	F-value			
Replication	4	5.21 **	1.81 ns	1.16 ns	11.49 **
Soil depth (S)	4	12.88 **	430.67 **	18.72 **	533.03 **
Time (T)	3	10.45 **	2.20 ns	14.17 **	1.14 ns
S x T	12	9.36 **	3.73 **	5.83 **	1.11 ns
Mean		0.007	0.087	0.006	0.078
C.V. (%)		5.46	9.52	5.21	6.95

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively; (1) data transformed with logarithm base 10; (2) data transformed with square root

Appendix 3: Effect of soil depth on atom% ^{15}N excess[1] in soil soil in Kaduwaa and Makmur at 0 and 28 days after injection of ^{15}N-Ammonium-^{15}N-Nitrate

Treatment	df	Jul-02	Aug-02	Dec1-02	Jan-03
		(atom% ^{15}N excess, Kaduwaa, F-value)			
Replication	2	0.65 ns	3.04 ns	7.15 *	0.91 ns
Depth	3	6.94 *	2.16 ns	10.33 **	1.81 ns
Mean		0.19	0.07	0.16	0.07
C.V. (%)		40.63	50.07	28.91	48.13
		(atom% ^{15}N excess, Makmur, F-value)			
Replication	2	0.18 ns	2.17 ns	0.04 ns	2.17 ns
Depth	3	4.42 *	0.28 ns	1.97 ns	0.28 ns
Mean		0.16	0.03	0.16	0.03
C.V. (%)		51.48	64.73	55.42	64.73

*[1]Data is taken only at the point of fertilizer injection; *, **, ns = significant at 0.05, 0.01 and non-significant, respectively (Jul-02 and Dec1-02= 0 day after injection; Aug-02 and Jan-03= 28 days after injection)*

Appendix 4: Effect of distance from the injection on atom% ^{15}N excess in soil in Kaduwaa at 0 and 28 days after injection of ^{15}N-Ammonium-^{15}N-Nitrate

Treatment		Jul-02	Aug-02	Dec1-02	Jan-03
	df				
		atom% ^{15}N excess(1), F-value			
Replication	2	0.00 ns	0.55 ns	4.21 *	1.87 ns
Depth (D)	3	5.56 **	3.04 ns	16.65 **	2.22 ns
Distance (S)	2	9.87 **	9.87 **	110.75 **	193.27 **
DxS	6	0.61 ns	0.58 ns	5.68 **	1.10 ns
Mean		0.11	0.03	0.07	0.03
C.V. (%)		52.52	45.82	57.57	41.59

**, **, ns = significant at 0.05, 0.01 and non-significant, respectively (Jul-02 and Dec1-02= 0 day after injection; Aug-02 and Jan-03= 28 days after injection); (1) data transformed with logarithm base 10*

Appendix 5: Effect of soil depth in enrichment plots on plant-available soil N and ^{15}N

Treatment	df	NH_4^+	$^{15}NH4^{+(1)}$	df	NO_3^-	$^{15}NO_3^{-(1)}$
		---------- F-Value----------				
Replication	2	4.05 *	3.09 ns	2	1.20 ns	1.96 ns
Site (S)	1	0.28 ns	4.81 *	1	2.60 ns	6.88 *
Soil depth (D)	4	49.90 **	0.42 ns	3	11.16 **	0.48 ns
S x D	4	1.62 ns	0.19 ns	3	1.04 ns	0.32 ns
Mean		5.58	0.23		3.32	0.21
C.V. (%)		22.94	45.62		54.13	21.57

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively; (1) data transformed with square root

Appendix 6: Effect of plant species and time of sampling on atom% ^{15}N excess and $\%N_{tot}$ in leaves, twigs and litter in Kaduwaa

Treatment	Leaves			Twigs			Litter		
		Atom% ^{15}N excess	N_{tot} (%)		Atom% ^{15}N excess	N_{tot} (%)		Atom% ^{15}N excess	N_{tot} (%)
	df	F-value		df	F-value		df	F-value	
Replication	4	1.99ns	0.83ns	4	0.99ns	3.68ns	4	1.17ns	3.70*
Plant (P)	2	36.68**	145.55**	2	9.05**	35.46**	2	38.85**	399.8**
Time (T)	3	22.67**	23.86**				3	34.51**	2.39ns
P x T	6	4.28**	8.63**				6	3.74**	8.43**
Mean		0.15	3.06		0.16	1.47		0.10	1.90
C.V. (%)		12.99	9.31		14.29	11.86		11.42	10.49

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 7: Effect of plant species and time of sampling on atom% ^{15}N excess and $\%N_{tot}$ in leaves, twigs and litter in Makmur

Treatment	Leaves			Twigs			Litter		
		Atom% ^{15}N excess	N_{tot} (%)		Atom% ^{15}N excess	N_{tot} (%)		Atom% ^{15}N excess	N_{tot} (%)
	df	F-value		df	F-value		df	F-value	
Replication	4	1.58ns	1.09ns	4	3.84*	1.96ns	4	0.32ns	3.64*
Plant (P)	3	20.30**	239.8**	2	8.71**	17.32**	1	84.86**	9.33**
Time (T)	3	23.05**	27.23**				3	10.78**	6.50**
P x T	9	1.64ns	4.82**				3	5.34**	4.44*
Mean		0.13	2.58		0.21	1.25		0.14	1.96
C.V. (%)		14.29	12.68		18.73	19.25		12.28	17.95

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 8: Atom% ^{15}N excess in litter of fixing and reference plants at different times of sampling in Kaduwaa and Makmur

Time of sampling	Kaduwaa			Makmur	
	Gliricidia	Cacao	Coffee	Gliricidia	Cacao
	Atom% ^{15}N excess				
Oct-02	0.015 (0.02)	0.044 (0.01)	0.056 (0.01)	0.087 (0.02)	0.304 (0.02)
Dec-02	0.039 (0.00)	0.061 (0.02)	0.104 (0.01)	0.065 (0.00)	0.143 (0.03)
Mar-03	0.079 (0.01)	0.120 (0.02)	0.189 (0.03)	0.098 (0.01)	0.271 (0.03)
Jun-03	0.070 (0.01)	0.145 (0.03)	0.249 (0.03)	0.067 (0.01)	0.148 (0.01)
Mean	0.508 (0.01)	0.927 (0.01)	0.150 (0.02)	0.079 (0.01)	0.212 (0.02)
	N_{tot} (%)				
Oct-02	2.17 (0.06)	1.42 (0.10)	2.24 (0.09)	1.95 (0.11)	2.54 (0.35)
Dec-02	2.18 (0.05)	1.25 (0.05)	2.14 (0.06)	2.08 (0.20)	1.86 (0.09)
Mar-03	2.42 (0.05)	0.98 (0.06)	2.42 (0.12)	1.54 (0.07)	1.94 (0.13)
Jun-03	2.30 (0.03)	0.94 (0.06)	2.25 (0.07)	1.77 (0.08)	2.01 (0.10)
Mean	2.27 (0.03)	1.15 (0.06)	2.26 (0.05)	1.83 (0.06)	2.06 (0.10)

Value in brackets represents standard error of the means

Appendix 9: Effect of reference plants and time of sampling on proportion of N derived from atmospheric N_2 (%Ndfa) of Gliricidia determined with ^{15}N enrichment method

Treatment	Kaduwaa		Makmur		Kaduwaa		Makmur	
	Ndfa (%)							
	Leaves				Litter			
	Df	F-value	df	F-value	df	F-value	df	F-value
Replication	4	6.51 **	4	0.58 ns	4	2.22 ns	4	3.28 ns
Plant (P)	1	0.78 ns	2	10.01 **	1	12.35 **		
Time (T)	3	4.77 **	3	1.21 ns	3	2.64 ns	3	1.93 ns
P x T	3	0.54 ns	6	1.54 ns	3	0.26 ns		
Mean	55.93		43.72		51.32		55.63	
C.V. (%)	27.06		46.16		36.92		29.50	

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 10: Effect of soil depth and time of sampling on $\delta^{15}N$ value and $\%N_{tot}$ in soil in Kaduwaa and Makmur

Treatment		Kaduwaa		Makmur	
		$\delta^{15}N$ (‰)$^{(1)}$	N_{tot} (%)$^{(2)}$	$\delta^{15}N$ (‰)$^{(1)}$	N_{tot} (%)$^{(2)}$
	df	F-value			
Replication	4	1.66 ns	2.35 ns	1.25 ns	0.94 ns
Soil depth (S)	4	3.21 **	316.09 **	16.58 **	79.12 **
Time (T)	4	30.73 **	12.70 **	97.88 **	5.80 **
S x T	16	6.38 **	1.74 ns	6.34 **	1.07 ns
Mean		0.90	0.10	0.85	0.28
C.V. (%)		2.27	23.24	5.68	16.03

**, **, ns = significant at 0.05, 0.01 and non-significant, respectively; (1) data transformed with logarithm base 10; (2) data transformed with square root*

Appendix 11: Effect of soil taken from different depths on $\delta^{15}N$ value of *Oryza sativa* shoots

Treatment	df	$\delta^{15}N$ (‰)
Site (S)	1	18.82 **
Soil depth (D)	4	4.21**
S x D	4	0.86 ns
Mean		5.14
C.V. (%)		12.17

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 12: Effect of soil depth in natural abundance plots on plant-available soil N and ^{15}N

Treatment	df	NH_4^+	$^{15}NH4^+$	df	NO_3^-	$^{15}NO_3^-$
		F-Value				
Replication	2	4.92 *	6.37 ns	2	2.57 ns	1.35 ns
Site (S)	1	0.60 ns	47.41 **	1	1.12 ns	0.61 ns
Soil depth (D)	4	42.91 **	2.03 ns	3	4.69 *	0.64 ns
S x D	4	1.09 ns	0.95 ns	3	1.34 ns	0.28 ns
Mean		4.45	0.08		3.97	4.79
C.V. (%)		27.98	24.69		58.34	6.97

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 13: Effect of plant species and time of sampling on $\delta^{15}N$ and $\%N_{tot}$ in leaves, twigs and litter in Kaduwaa

Treatment		Leaves			Twigs			Litter	
		$\delta^{15}N$	N_{tot}		$\delta^{15}N$	N_{tot}		$\delta^{15}N$	N_{tot}
	df	F-value		df	F-value		df	F-value	
Replication	4	1.59 ns	0.32 ns	4	2.68 ns	0.46 ns	4	1.13ns	2.55*
Plants (P)	2	94.66**	218.34**	2	37.73**	29.86**	2	42.79**	225.1**
Time (T)	4	62.44**	17.34**	1	0.40ns	25.64**	4	12.60**	2.95*
P x T	8	11.24**	5.14**	2	3.03ns	16.03**	8	12.28**	2.86*
Mean		4.82	3.09		3.89	1.43		3.91	1.94
C.V. (%)		12.66	9.85		19.00	18.75		14.93	9.32

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 14: Effect of plant species and time of sampling on $\delta^{15}N$ and $\%N_{tot}$ in leaves, twigs and litter in Makmur

Treatment		Leaves			Twigs			Litter	
		$\delta^{15}N$	N_{tot}		$\delta^{15}N$	N_{tot}		$\delta^{15}N$	N_{tot}
	df	F-value		df	F-value		df	F-value	
Replication	4	3.02 ns	1.16ns	4	2.58 ns	0.70ns	4	0.86*	0.71ns
Plant (P)	3	58.79**	369.4**	2	29.97**	5.05*	1	41.92**	127.6**
Time (T)	4	9.02**	49.90**	1	17.35**	0.19ns	4	9.18**	3.56*
P x T	12	6.92**	20.99**	2	12.31**	0.72ns	4	4.68**	61.90**
Mean		3.83	2.32		2.00	1.38		3.31	1.50
C.V. (%)		26.85	11.64		41.46	15.78		29.24	10.45

**, **, ns = significant at 0.05, 0.01 and non-significant, respectively*

Appendix 15: $\delta^{15}N$ value and $\%N_{tot}$ in twigs of fixing and reference plants at different times of sampling in Kaduwaa

Time of sampling	Kaduwaa			Makmur		
	Gliricidia	Cacao	Coffee	Gliricidia	Cacao	Sida
			$\delta^{15}N$ (‰)			
Jul-02	1.94 (0.07)	4.04 (0.42)	5.47 (0.56)	-0.03 (0.20)	0.28 (0.30)	3.86 (0.35)
Oct-02	2.26 (0.05)	4.59 (0.38)	4.70 (0.26)	1.40 (0.17)	3.29 (0.36)	3.53 (0.64)
Mean	3.10 (0.46)	4.31 (0.28)	5.08 (0.32)	0.68 (0.27)	1.78 (0.55)	3.53 (0.42)
			N_{tot} (%)			
Jul-02	1.11 (0.17)	0.81 (0.13)	1.63 (0.07)	1.71 (0.07)	0.85 (0.04)	1.44 (0.09)
Oct-02	2.39 (0.23)	0.99 (0.06)	1.66 (0.10)	0.91 (0.07)	1.66 (0.13)	1.88 (0.13)
Mean	1.75 (0.24)	0.90 (0.06)	1.64 (0.06)	1.83 (0.06)	2.06 (0.10)	1.66 (0.31)

Value in brackets represents standard error of means

Appendix 16: $\delta^{15}N$ value and $\%N_{tot}$ in litter of fixing and reference plants at different times of sampling in Makmur

Time of sampling	Kaduwaa			Makmur	
	Gliricidia	Cacao	Coffee	Gliricidia	Cacao
			$\delta^{15}N$ (‰)		
Jul-02	3.43 (0.36)	4.15 (0.43)	5.81 (0.18)	0.96 (0.34)	4.00 (0.51)
Oct-02	4.35 (0.34)	4.53 (0.36)	4.50 (0.15)	4.05 (0.23)	4.54 (0.45)
Dec-02	1.52 (0.32)	4.84 (0.22)	3.37 (0.10)	3.32 (0.92)	5.06 (0.23)
Mar-03	2.53 (0.32)	5.03 (0.28)	2.94 (0.29)	1.61 (0.24)	5.18 (0.27)
Jun-03	3.12 (0.14)	3.63 (0.18)	4.74 (0.15)	1.92 (0.24)	2.40 (0.19)
Mean	2.99 (0.22)	4.42 (0.17)	4.27 (0.22)	2.25 (0.32)	4.24 (0.25)
			N_{tot} (%)		
Jul-02	2.13 (0.13)	1.38 (0.12)	2.52 (0.06)	1.01 (0.06)	1.80 (0.09)
Oct-02	2.31 (0.07)	1.45 (0.12)	2.08 (0.03)	1.67 (0.08)	1.35 (0.04)
Dec-02	2.13 (0.06)	1.23 (0.09)	2.21 (0.04)	2.17 (0.04)	1.13 (0.11)
Mar-03	2.42 (0.12)	1.17 (0.11)	2.31 (0.05)	1.96 (0.10)	0.99 (0.04)
Jun-03	2.42 (0.06)	1.35 (0.05)	2.33 (0.05)	1.92 (0.04)	0.96 (0.04)
Mean	2.21 (0.05)	1.32 (0.05)	2.29 (0.04)	1.72 (0.09)	1.25 (0.07)

Value in brackets represents standard error of means

Appendix 17: Effect of type of soil solution and plant part on dry matter, total N accumulation and $\delta^{15}N$ value of Gliricidia at different times of harvest grown in pure sand and irrigated with a N-free solution under screenhouse condition 12, 24 and 36 WAP

Treatments	df	Dry matter F-value	N accumulation F-value	$\delta^{15}N^{(1)}$ F-value
			12WAP	
Soil solution (S)	1	0.00 ns	0.14 ns	1.21 ns
Plant part (P)	4	53.22 **	52.30 **	176.45 **
SxP	4	0.23 ns	0.25 ns	0.57 ns
Mean		1.69	0.33	0.23
C.V. (%)		27.75	29.45	17.17
			24WAP	
Soil solution (S)	1	0.63 ns	0.67 ns	0.20 ns
Plant part (P)	4	138.77 **	174.74 **	42.87 **
SxP	4	0.11 ns	0.92 ns	0.82 ns
Mean		6.10	0.10	0.73
C.V. (%)		22.70	16.36	23.69
			36WAP	
Soil solution (S)	1	0.01 ns	0.51 ns	1.45 ns
Plant parts (P)	4	116.10 **	65.18 **	109.74 **
SxP	4	0.48 ns	0.71 ns	0.73 ns
Mean		15.23	0.25	0.75
C.V. (%)		22.76	24.00	23.85

**, **, ns = significant at 0.05, 0.01 and non-significant, respectively; WAP= weeks after planting; (1) data transformed with log base 10*

Appendix 18: Effect of plant species and time of sampling on proportion of N derived from atmospheric N_2 (%Ndfa) by Gliricidia determined with ^{15}N natural abundance method

	Ndfa (%)							
	Kaduwaa				Makmur			
		Leaves	Twigs	Litter		Leaves	Twigs	Litter
	df	F-value			df	F-value		
Replication	4	1.47 ns	4.42 *	1.89 ns	4	0.69 ns	1.39 ns	1.56 ns
Plant (P)	1	0.51 ns	1.32 ns	0.32 ns	2	8.98 **	7.06 *	
Time (T)	4	25.22 **	36.73 **	11.21 **	4	9.62 **	0.85 ns	7.36 **
P x T	4	4.03 **	0.54 ns	6.59 **	8	2.32 *	9.53 **	
Mean		32.65	27.67	24.83		39.32	49.11	36.81
C.V. (%)		29.91	59.36	59.77		41.04	50.83	55.58

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively

Appendix 19: Effect of soil type and legume crop as plant trapping indicator on nodule fresh weight, nodule number and infection potential of soil in Kaduwaa and Makmur

Treatment	df	Nodule fresh F-value	Nodule number F-value	Infection potential F-value
Soil type (S)	1	38.37 **	41.43 **	41.42 **
Crop (C)	1	18.84 **	14.24 **	14.25 **
SxC	1	12.16 **	13.41 **	13.38 **
Mean		30.65	81.33	2.71
C.V. (%)		35.67	25.59	25.61

**, **, ns = significant at 0.05, 0.01 and non-significant, respectively*

Appendix 20: Effect of site and time of sampling on total dry matter and N accumulation of Gliricidia litter

Treatment	df	Dry matter ($g\ m^{-2}$)(1)	N accumulation ($g\ m^{-2}$)(2)
		----------F value----------	
Replication	4	2.79 *	3.02 *
Site (S)	1	19.67 **	0.01 ns
Time (T)	11	24.55 **	27.36 **
S x T	11	11.16 **	12.89 **
Mean		1.39	0.72
C.V. (%)		7.67	11.93

*, **, ns = significant at 0.05, 0.01 and non-significant, respectively; (1) data transformed with logarithm base 10; (2) data transformed with square root

Appendix 21: Dry matter aboveground biomass of 8 Gliricidia in cacao agroforestry stands in Kaduwaa and Makmur

Tree	Diameter (cm)	Leaflets	Twigs (Ø, < 2)	Branches (2<Ø< 5)	Large branches and stems (Ø >2)	Total
				($kg\ tree^{-1}$)		
1	6.63	0.93	3.16	6.85	3.81	14.75
2	7.88	1.26	5.06	12.21	6.15	24.68
3	9.88	1.15	7.76	11.85	12.31	33.07
4	10.00	1.85	9.1	10.76	13.54	35.25
5	10.25	2.49	12.89	18.34	12.71	46.43
6	10.75	1.62	11.71	16.6	20.53	50.46
7	11.63	1.96	6.46	20.31	18.27	47.00
8	14.38	5.54	14.61	29.39	35.65	85.19
Average	10.17	2.10	8.84	15.79	15.37	42.10
% of total		4.99	21.00	37.50	36.51	

Ø = basal diameter

Appendix 22: Total N accumulation and N concentration of 8 Gliricidia in cacao agroforestry stand in Kaduwaa and Makmur

Tree	Diameter (cm)	Leaflets	Twigs (Ø<2cm)	Branches (2<Ø< 5)	Large branches and stems (Ø>5cm)	Total N	Total N (%)
				(g tree^{-1})			
1	6.63	36.83	52.93	79.73	24.00	193.5	1.31
2	7.88	49.90	84.76	142.12	38.75	315.5	1.28
3	9.88	45.54	129.98	137.93	77.55	391.0	1.18
4	10.00	73.26	152.43	125.25	85.30	436.2	1.24
5	10.25	98.60	215.91	213.48	80.07	608.1	1.31
6	10.75	64.15	196.14	193.22	129.34	582.9	1.16
7	11.63	77.62	108.21	236.41	115.10	537.3	1.14
8	14.38	219.38	244.72	342.10	224.60	1030.8	1.21
Average	10.17	83.16	148.13	183.78	96.84	511.91	1.22
% of total		16.24	28.94	35.90	18.92		

Ø = basal diameter; Leaflets N_{tot}= 0.0396%, Twigs N_{tot}= 0.0167%, Branches N_{tot}= 0, 0.0116%, Large branches and stems N_{tot}= 0.0063%

ACKNOWLEDGEMENTS

I am very grateful to Prof. Dr. Paul Vlek, my first supervisor, for his guidance, support and encouragement at all stages of my work. I also thank Prof. Dr. Mathias Becker, my second supervisor, for his advice and for sharing his ideas on the manuscript. I could not have completed this disertation without the generous help of Dr. Rolf Sommer, who gives support, suggestion, and constructive comments on the manuscript. His technical and editorial advice greatly improve the content of this disertation. For this, I am very grateful.

I would like to express my gratitude to DAAD for giving me the financial support during my study in Germany and my field research in Palu, Indonesia. I thank the Zentrum für Entwicklungsforshung (ZEF) of the University of Bonn for hosting me for throughout the period of this study.

Many thanks to Dr. Eric T. Craswell and Dr. Manfred Denich for giving valuable technical and profesional advice. Special thanks also go to Dr. Guenther Manske for helping me with many adminstrative matters and for visiting my field site in Palu, Indonesia.

I also wish to thank Ms. Hanna Petters, Ms.Sabine Aengenendt-Baer, Ms. Miryam Schneider and Ms.Andrea Berg of ZEF for their office asisstant and kind help, Ms. Deborah Rupprecht for analyzing my samples for ^{15}N in Bonn, Dr. Reiner Brumme, Ms. Edit Nadia and Dr. Marife Corre for mineral ^{15}N analyses in Goettingen, and Ms. Margaret Jend for editing my disertation draft and translating my abstract and summary into German

I thank all the STORMA colleagues for their helpf in many ways during my stay in Palu, Indonesia, especially to Mr. Joko purbopuspito and Mr. Georg Dechert. My thank also goes to the STORMA coordination team in Palu, Bogor, and Göttingen. I thank Ms. Rina Yusuf, Mr. Robert Karsten, the laboratory staffs, and all drivers of the project in Palu for their support. I am very grateful to Mr. Budi and Mr. Iskandar, the farmers, for allowing me to use their farms for the study, and to Mr. M. Ikbal, Mr, Mesak, and Mr. Edi, who are my filed and laboratory asisstants in Palu.

Special thanks go to my collegues Mr. Quang Bao Le, Mr. Mekuria Argaw, Mr. Syahrinudin, Mr. Lulseged Idesta, Mr. Pepijn Screinemachers, Ms. Prasnee Tipragsa, Ms. Raquel Lopez, Ms. Sanaa Ashour, Ms. Kavita Rai, Ms. Dennis Aviles, Ms. Christina Carambas, Ms, Charlote van der Schaaf, Mr. Thomas Bagamsa and Ms. Almut Brunner for wonderful time, support and encouragement during our stay at ZEF. Also thanks to all Indonesien family in Bonn and Köln, many other friends and individuals, though not mentioned here, for their role in making my study succesful.

My sincere gratitude goes to my family, my parents – late Waliuddin Lubis and Siti Rahma Nasution, my sisters – late Nurmadiah Lubis and Masidah Hanum Lubis for their prayers, lovings and moral support; to my uncle and aunt – late M. Yunus Lubis and late Nurlela Siregar, who have given all their best for my education and my future; and to my brothers – Khairul A. Lubis and late Khairul S. Lubis, my sisters – Fauziah A. Fitri Lubis and Yanti M.S. Lubis, I thank for their support.

Finally, I sincerely thank my loving wife Rama Herawaty, for standing by me throughout the years. Her prayer, support, care and patience have immensely contributed to my acheivement. I thank our children Ismail Saleh Lubis, Raash Abdurrahman Lubis, and Nurul Hanifah Lubis, for being a source of inspirations.

Above all, thanks to Allah S.W.T., for all the blessings he has showered me.