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REGULAR ARTICLE

The contribution of *Stylosanthes guianensis* to the nitrogen cycle in a low input legume-rice rotation under conservation agriculture

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Abstract

Background and aims Legumes integrated in crop rotations are intended to improve crop nitrogen (N) supply and yield. In conservation agriculture (CA) systems under low input conditions on highly weathered tropical soils, experimental evidence for these benefits is lacking. To understand the mechanisms and evaluate the impact of the legume N on the subsequent crop, an in-depth study on N dynamics in the soil-plant system was conducted.

Methods In Madagascar, a CA based crop rotation with the perennial forage legume *Stylosanthes guianensis* (stylo) and upland rice (rice/stylo – stylo - rice/stylo) was established under three fertilization regimes. In addition, rice was grown in a non-CA bare fallow rotation without fertilizer. Over the three years N_2 fixed in stylo shoots, the incorporation of stylo shoot (mulch) N into soil N pools and mulch N uptake by rice was quantified using ^{15}N techniques and mulch and stylo root residue decomposition was investigated in a litterbag study.

Results N_2 fixed in stylo shoots ranged from 96 to 122 kg N ha^{-1}. Between 50 to 70% of stylo mulch and root residues decomposed during the third cropping season. Without fertilizer, grain yield of rice after the fallow with stylo was about 70% greater than after bare fallow, corresponding to 11 kg N ha^{-1} greater N uptake. Recoveries of stylo mulch N after rice harvest were on average 64% in soil, with about 3% in each of the microbial and mineral N pools, with 39% on the soil surface, and 6% in the rice crop. The N input via stylo seed, leaf litter and belowground N totalled about three times the amount of N contained in stylo mulch, which usually is considered as major rice N source.

Conclusions Legumes, like stylo, can improve crop N supply and yield in low input CA cropping systems on highly weathered tropical soils. To explain the impact and mechanisms involved requires a consideration of all legume-N components beyond the mulch N present at the onset of the rice-cropping season.

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Abbreviations

CA	Conservation agriculture
CA-NON	CA based upland rice - stylo fallow rotation were rice received no fertilizer
CA-FYM	CA based upland rice - stylo fallow rotation were rice received farmyard manure
CA-MIN	CA based upland rice - stylo fallow rotation were rice received mineral fertilizer

DM	Dry matter
MC-NON	Mono cropped upland rice - bare fallow rotation were rice received no fertilizer
%Ndfa	Proportion of total N derived from the fixation of atmospheric N_2
%Ndfm	Proportion of N derived from stylo mulch N in a plant or soil N pool
SD	Standard deviation

Introduction

A sustainable yield increase on highly weathered soils in low input cropping systems of the tropics requires management practices that maximize the use of available resources, control pest and diseases (Médiène et al. 2011) and reverse soil fertility decline to improve crop nutrient supply, especially nitrogen (N) (Lal and Stewart 2014). An approach that may contribute to achieve this goal is conservation agriculture (CA) including legumes. Conservation agriculture is based on three agronomic principles; minimum mechanical soil disturbance, permanent organic soil cover and the use of diverse crop rotations and/or associations (Hobbs et al. 2008). Gains in crop N supply under CA are intended to occur firstly, as a result of a decrease in N losses from leaching, runoff and erosion through soil protective measures and/or secondly, through an increased N input through N_2 fixation when legumes are included in crop rotations (Fageria et al. 2005; FAO 2011). On the other hand, surface application of mulch can result in increased gaseous N losses (Six et al. 2004). Along with gaseous N losses, immobilization of N in surface residues and decreased N mineralization rates can limit N availability and potentially decrease yields in no-till (Thierfelder and Wall 2011). In industrialized countries CA farmers have learned to cope with such crop N supply shortages by N fertilizer application. However, for smallholders in the tropics this option my not be feasible considering the little use of fertilizer N (Chianu et al. 2012). Moreover, tropical edaphic conditions can constrain legume symbiotic N_2 fixation (Giller 2001). For instance, soil acidity, calcium, magnesium, molybdenum or phosphorus deficiency can impair rhizobia survival (Hartwig 1998).

Although the potential challenges of CA are known, the role of legume N in CA established in tropical low input cropping systems on highly weathered soils remains unclear. Most studies on CA including legumes in this context have focused on maize - grain legume rotations and the effects of soil management, i.e., tillage vs. no till, on soil and water conservation and crop yields (Nyagumbo et al. 2016; Rusinamhodzi et al. 2011; Thierfelder et al. 2013). Investigations that addressed N dynamics in such a CA system and obtained positive effects on crop N uptake and yield (e.g., Maltas et al. (2009); Mhlanga et al. (2015)) could not conclusively relate these effects to the N input from the legume, because they did not differentiate between crop N derived from the soil or the legume. The use of ^{15}N isotope techniques, which allow following the fate of residue N in the soil and quantifying its uptake by the subsequent crop (Hauck and Bremner 1976; Hood et al. 2008) combined with measurements of legume N_2 fixation, and of residual N in the soil could improve our understanding of benefits from legume N in these CA systems.

Experiments conducted in Madagascar to increase crop yield with little external inputs on highly weathered soils led to the introduction of the perennial forage legume *Stylosanthes guianensis* (stylo) into a CA crop rotation with upland rice (Husson et al. 2006). In this system, stylo is established as a relay crop with rice, growing simultaneously between rice rows. After rice harvest, stylo continues growing. Stylo shoot biomass is then cut and applied as mulch and the next rice crop is directly sown into the mulch. Yield improvements have been reported for rice and maize under CA with stylo after six years of conversion compared to yields under conventional practice in field experiments in the Midwest of Madagascar, but were mainly explained by lower weed (*Striga asiatica*) infestation under CA (Michellon et al. 2011). However, no study exists that follows symbiotic N_2 fixation by stylo and the transfer of stylo N to soil and the subsequent crop to show the assumed improvement in crop N supply after stylo fallow in this system.

Indeed, although stylo belongs to the tropical forge legume species most cited in the literature from sub-Saharan Africa (Agishi 1991; Thomas and Sumberg 1995), its N effect remains poorly quantified and few publications address the effect of stylo on rice N supply when used as a rotation crop in rainfed rice cropping systems. For example, stylo as a short term (four to six months) crop grown in Sub-Saharan Africa produced 1 to 17 Mg ha^{-1}

of shoot dry matter (DM) and took up 40 to 200 kg N ha^{-1} of which 63 to 79% was derived from the atmosphere, depending on rainfall and the soil fertility status (Akanvou et al. 2002; Becker and Johnson 1998; Ojiem et al. 2007; Saito et al. 2010). In the savannahs of West Africa, rice yields following stylo increased on average by 79% (Becker and Johnson 1998) and 41% (Saito et al. 2010) as compared to a weedy fallow, while Akanvou et al. (2002) reported no significant effect on subsequent rice yields. The reasons for such contrasting crop yield responses are not clear. According to Becker and Johnson (1998) rice grain yield was positively correlated with the N accumulation in stylo, which was greater where stylo biomass was incorporated into the soil than where stylo biomass was burnt prior to rice sowing. However, although these authors used a ^{15}N isotope technique to determine the amount of N_2 fixed in stylo, they made no further use of ^{15}N techniques to trace the fate of residue N in the soil and its uptake by the subsequent crop. Indeed, reported research results were mostly incomplete, making it difficult to relate stylo residue N to rice yield. For example, none of the studies presented rice N uptake, some even did not report the actual N in stylo biomass that was applied. Only Saito et al. (2010) measured the total and mineral soil N pool in the subsequent rice-cropping season, as an additional indicator for stylo N supply to rice, but found no significant effects of fallow and tillage management on these pools. Lastly, none of the studies quantified stylo root biomass, which is considered a substantial N source from legume residues (Peoples et al. 2001) and none of them studied stylo contribution to rice N supply under CA management (i.e., with no tillage and superficial legume residue restitution).

An overall picture on the supply of legume N to crops grown under CA, including the soil N dynamics as affected by the integration of legumes in CA, is lacking. To enhance our understanding on legume N supply to rice in low input tropical upland cropping systems under CA we established a field trial on a highly weathered Ferralsol in Madagascar. We used ^{15}N isotope techniques to study how stylo grown as a relay crop in a CA based rice cropping system affects under different fertilizer regimes (1) the amount of N_2 fixed in stylo, (2) the rates of stylo mulch and root residue decomposition and N release and (3) the recovery of stylo mulch N in soil N pools and the succeeding rice crop.

Materials and methods

Study site description

The experimental site was located next to the village of Ivory (19°33′36.1” S, 46°24′34.7″ E) at an altitude of 932 m in the region of Vakinankaratra, Madagascar. It was established on an arable field of approximately 0.5 ha, which had been hitherto farmer-managed and used for cropping. Subsistence agriculture is the main activity in this densely populated area of the Midwest Highlands. Farmers typically have about 1 ha of lowland where they grow irrigated rice and 0.5 to 2 ha of upland called `*tanety*` for rainfed crop production. Most farmers have also a few cattle (*Bos indicus*) used as draught animals. According to Koppen-Geiger classification (Peel et al. 2007), the climatic conditions correspond to a tropical savannah climate. On average the region receives 1300 mm of rain per year and the mean annual temperature is 23 °C. Annual rainfall has a wide year-to-year variation (decennial (2004–14) annual rainfall range 870 to 1800 mm) due to tropical cyclone and storm formations, which mainly hit Madagascar during the cyclone period from January to March (WMO 2015). Usually, the first rains fall erratically in mid-October and November, often followed by a short dry spell, before the main rainfall occurs from December to March (cropping season) and again erratic rains in April and May mark the end of the rainy season. During the field trial the total annual rainfall was 1800 mm (2010–11), 980 mm (2011–12) and 1450 mm (2012–13) (Fig. 1). The soil has been classified as haplic Ferralsol (Jones et al. 2013; WRB 2014) and has a sandy clay loam texture with 26% clay, 25% silt and 49% sand in the top soil layer (0 cm to 20 cm). Total N, phosphorus (P) and organic carbon concentration in the topsoil were 0.8, 0.4 and 10.8 g kg^{-1} soil and the soil pH (H_2O) was 4.9 when the field experiment started.

Experimental design and treatments

The field trial was arranged as a completely randomized block experiment with four replicates. Treatment plot size was 6 m × 10 m (hereafter referred to as main plots). In the crop rotation upland rice (NERICA 4, WAB 450-I-B-P-91-HB) was relay cropped with the forage legume *Stylosanthes guianensis cv.* Pucallpa (CIAT 184) (stylo) in cropping season one (Dec. 2010 to Mar. 2011), followed by one and a half year of stylo (Apr. 2011 to

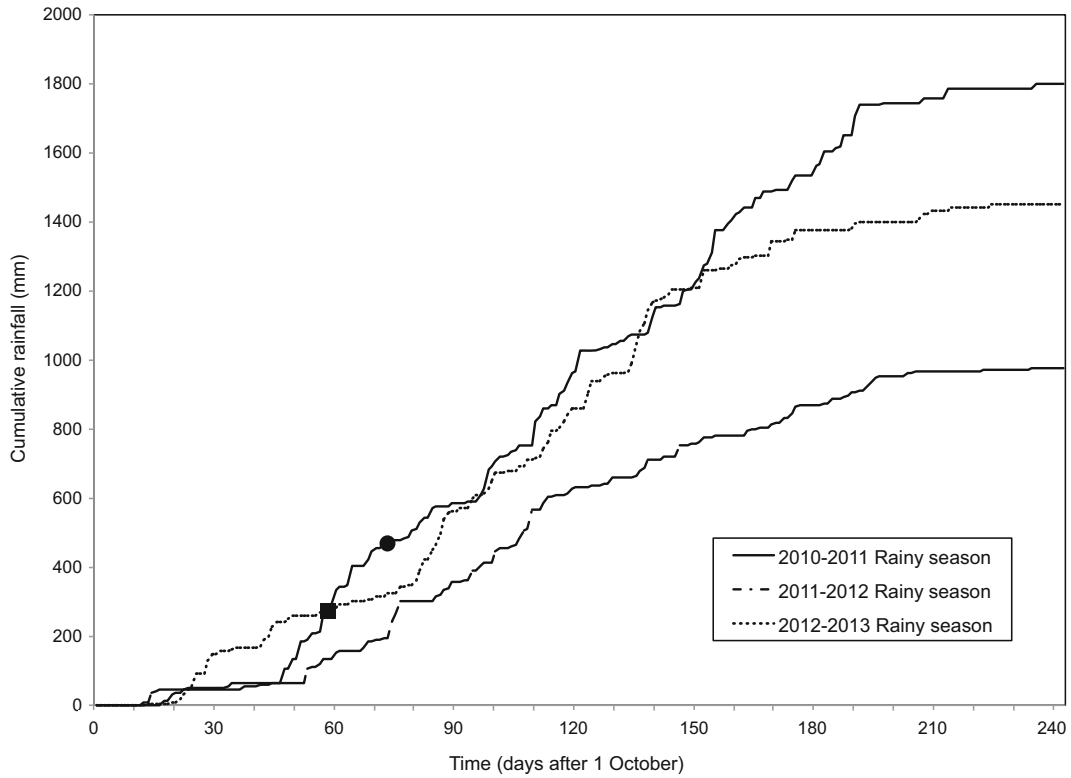

Fig. 1 Cumulative rainfall (mm) during the first (2010–11), second (2011–12) and third (2012–13) rainy season at the field trial site next to the village of Ivory, Madagascar. The black square indicates the sowing date of the rice crop in the first and third rainy season, while the black dot shows the date during the first rainy season when stylo was introduced into the standing rice crop

Oct. 2012), before in the third cropping season (Dec. 2012 to Mar. 2013) rice was again relay cropped with stylo. Treatments in this CA rotation (rice/stylo – stylo - rice/stylo) comprised the application of no fertilizer (NON), farmyard manure (FYM) or mineral fertilizer (MIN) to rice (Fig. 2). The respective acronyms were CA-NON, CA-FYM and CA-MIN. In addition to the CA rotation, a non-CA treatment termed MC-NON was established as an indicator of general/indigenous soil fertility. In the MC-NON treatment rice was grown without fertilizer as a mono crop (MC) in cropping season one and three, while in between the treatment plots remained as bare fallow (Fig. 2).

Crop management

At the start (November 2010) soil in all plots was tilled prior to sowing. As commonly practiced in this study area, tillage was conducted by hand using a long bladed version of the traditional spade `*angady*`, which allows turning the soil down to a depth of 20 cm. In December 2010, five to seven rice seeds were sown by hand in pockets with an inter-row spacing of 20 cm and an intra-row spacing of 30 cm. Rice seeds were protected from insect attacks by treating the seeds with insecticide (Insector T 45 DS ®, 35% imidacloprid and 10% thirame at 4 g kg^{-1} seeds). Stylo seeds were sown 14 days after rice at a rate of 2 kg ha^{-1} in between the rice rows (spacing rice - stylo - rice: 15 cm) with an inter-row spacing of 40 cm between stylo pockets. This time lag of two weeks minimizes competition with upland rice while optimizing establishment and biomass accumulation of stylo (Shelton and Humphreys 1975). Seeding pockets for the rice and stylo seeds were prepared by using a narrow bladed version of the aforementioned angady as dibble stick. To break seed dormancy stylo seeds were submerged for 10 min in hot (80 °C) tap water (Nan et al. 1998). Neither soil liming, irrigation, nor rhizobia inoculation of stylo seeds or the soil were conducted, following

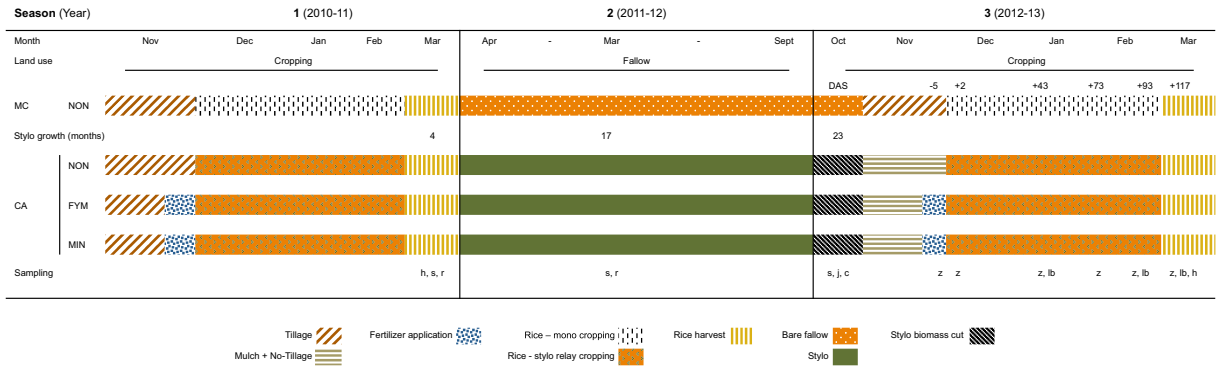

Fig. 2 Overview of the experimental timescale, crop - soil - fertilizer management and sampling activities: In the MC-NON treatment rice was mono cropped (MC) in tilled soil without fertiliser application (NON). In the conservation agriculture (CA) rotation stylo was established as a relay crop with rice and grew continuously for 23 months. Rice received no fertilizer (NON), farmyard manure (FYM) or NPK + urea (MIN) and was harvested (h) at the end of the first (2010–11) and third (2012–13) cropping season (December to March). Stylo shoot (s), root (r) and fallen litter (j) biomass was sampled as indicated. At the end of the second dry season (23 months) all aboveground stylo biomass was cut (c) at ground level and applied as mulch and rice was sown through the mulch into non-tilled soil. Litter bags (lb) with stylo shoot and root residues were sampled at 42, 92 and 118 days after their amendment. Soil sampling (z) of the 0 to 10 cm soil layer was conducted at −5, +2, +43, +73, +93 and +117 days after the sowing (DAS) of rice. At +117 DAS soil was addditionally sampled from the 10 to 20 and 20 to 30 cm soil layer

farmers practise. Rice received either no fertilizer, farmyard manure (5 Mg ha^{-1}) or mineral fertilizer as NPK (kg ha^{-1} 33 N, 29 P, 40 K) and urea (37 kg N ha^{-1}, at panicle initiation). Weeding was conducted manually 21 days after sowing and at panicle initiation of rice. At rice harvest (March 2011), rice straw was distributed on the soil surface after threshing and stylo continued to grow. Weeding was conducted in all plots once after the onset of the major rains (December 2011) to promote stylo growth and canopy closure. Prior to the start of the third cropping season (October 2012), i.e., 23 months after establishment, stylo shoot biomass was cut using an angady at ground level. All cut stylo aboveground biomass (including litter) was retained in the plots. To have the same amount of stylo shoot biomass in each plot, stylo shoot biomass (litter excluded) was adjusted to 9 Mg DM ha^{-1}. Additional shoot biomass was taken from plots with the same cropping history, but not designated for stylo residue retention (Zemek 2016). After 14 days of drying in the sun, stylo shoot biomass (litter excluded) was chopped into 10 cm to 15 cm large pieces using knifes (hereinafter referred to as stylo mulch) and uniformly distributed on the plot surface. In December 2012, rice was directly sown through stylo mulch using an angady as dibble stick. Stylo regenerated from soil seed bank as relay crop with rice. All other crop management practices were as described for season one.

Determination of stylo above- and belowground biomass production

Stylo shoot biomass was determined after four, 17 and 23 months of stylo growth (Fig. 2). To this end, stylo shoots from two times 1 m × 1 m per plot were cut at ground level. Litter (fallen leaves and inflorescences) was collected from the same area and cleaned from adhering soil particles at the sampling after the dry season in October 2012. Shoot biomass and litter from each sampled square was weighed and DM content determined by drying a subsample at 60 °C for 48 h.

Stylo roots were sampled at the end of the first and second cropping season (March 2011, 2012), when also shoot biomass was sampled (Fig. 2). Roots were sampled at 10 cm depth increments down to a soil depth of 30 cm. using an auger (Eijkelkamp, Giesbeek, the Netherlands, ø 8 cm). Soil cores were taken at four adjacent positions describing a rectangular pattern; i.e., directly above the cut plant and at half the inter-row, inter-line and inter-line-diagonal distance to the next plant. Roots were separated from soil by wet sieving, as described below. In season two, when stylo grew alone, stylo roots were sampled as follows: In each plot, at two different locations, one stylo plant was randomly selected. A frame of 40 cm × 40 cm was placed at equidistance (i.e., each side had a distance of 20 cm distance to the stem) around the stem of the selected stylo plant. The stylo plant shoot was cut and three soil monoliths of the

size 40 cm × 40 cm × 10 cm were consecutively excavated to a depth of 30 cm using a spade. Soil monoliths from the same sampling depth per plot were pooled, before roots and organic debris were separated from soil by sequential wet sieving (ø 5-, 2- and 0.5- mm mesh size). Roots retained in the different sieves were pooled and total root DM determined after drying the roots at 60 °C for 48 h. The proportions of stylo and rice roots in the retrieved root samples (average ratio 1:3) were determined following the DNA based molecular approaches of Linder et al. (2000) and Mommer et al. (2008).

Quantification of N_2 fixation by stylo

The proportion of N in stylo derived from the atmosphere (%Ndfa) was determined using the natural abundance method (Amarger et al. 1979). The method could be applied since the natural enrichment of ^{15}N in our soil relative to atmospheric N_2 was according to Cadisch et al. (2000) sufficiently high (10 to 12‰), with little variation in depth to 1.4 m (Supplementary information, Fig. S1) and in horizontal direction across all four blocks. Non-N_2 fixing reference plants comprised non-legume (mainly dicotyledonous) weeds and rice. For each sampled stylo plant, two weeds or, in the case of relay cropped stylo, one rice plant and one weed were chosen. Plant sampling was conducted towards the end of each cropping season (March), i.e., flowering to early seed setting of stylo (Fig. 2). In each of the four replicate plots per treatment, five stylo plants together with the two respective reference plants were harvested along a W-shaped pattern. All plants were cut at ground level and dried at 60 °C for 48 h.

Assessment of stylo residue decomposition and N release

The litterbag study conducted in the third cropping season (November 2012 – March 2013) was designed so that three litterbags were placed into each plot, corresponding to three sampling dates, i.e., 42, 92 and 118 days after their amendment. Stylo mulch litterbags were placed in horizontal position between rice rows on the soil surface under a layer of stylo mulch on soil surface. Root litterbags were buried at random locations in the soil in an upright position, spanning from 0 cm to 30 cm soil depth. Treatments included all CA-treatments, resulting in a total number of 72 litterbags. Stylo residues were derived at the end of the dry season from stylo plants, which had been continually grown in the field for 23 months (Fig. 2). Both residue types were dried at 60 °C for 48 h prior to use and subsamples were taken to determine their initial chemical composition (Table 1). In total 18 g of dried stylo mulch with a N concentration of 8 g N kg^{-1} DM was placed inside each litterbag (20 cm × 20 cm nylon bags, mesh size 1.5 mm). Bag dimensions and sample size corresponded to a stylo mulch application rate of 4.5 Mg DM ha^{-1} and 37 kg N ha^{-1}, i.e., half the total amount of stylo mulch applied in plots. For stylo root residues a representative portion of 10 g root DM with a N concentration of 10 g N kg^{-1} DM was placed into each litterbag (8 cm × 30 cm). Amounts of stylo root DM were based on field observations made during root excavation and corresponded to a stylo root application rate of 1 Mg DM ha^{-1} and 10 kg N ha^{-1}. Upon litterbag removal, roots that had grown into the bags were removed by handpicking. After opening the bags stylo mulch or root residue DM was briefly rinsed with tap water to remove any adhering soil particles. Residues were dried at 60 °C for 48 h and weighed to determine the percent mass loss. To correct the weight of the plant residues sampled for C and N analysis for contamination with soil, subsamples (500 mg) were incinerated at 550 °C for 4 h to determine the ash-free dry mass content.

Assessment of stylo mulch N recovery by rice

To quantify stylo mulch N recovery by rice and in soil N pools, ^{15}N labelled stylo was applied in micro plots (1.8 m × 1.6 m) confined with rectangular metal sheet frames that were installed to a depth of 30 cm within the respective main plots. The ^{15}N enriched stylo mulch was produced during the fallow season (2011–12). Therefore, 60 atom% ^{15}N enriched $(NH_4)_2SO_4$ was dissolved in tap water and applied at a rate of 9 kg N ha^{-1} to the soil surface between the stylo plants with a watering can. Simultaneously, sucrose (C:N ratio of 10:1) was applied, in order to promote homogenous soil N labelling through microbial turnover of the applied label (Hood et al. 2000). Following Douxchamps et al. (2011) to minimize leaching by the heavy rains the applied dose was split. The first dose was given at the start of the second cropping season (December 2011) and the second dose was applied three months later (February 2012). The resulting ^{15}N abundance of stylo N was 0.68 atom%. These stylo residues were then used

Table 1 Initial quantities and chemical composition of stylo residue dry matter (DM) placed in litterbags and corresponding residue application rates per m^{-2}

Residue type	DM g litterbag^{-1}	N conc. mg N g^{-1} DM	C conc. mg C g^{-1} DM	C:N mass ratio	DM¶ g DM m^{-2}	N applied¶ g N m^{-2}
Mulch†	18	8.3 (0.64)	451 (18.2)	55	450	3.7
Root§	10	10.2 (0.89)	447 (18.5)	44	100	1.0

Means ($n = 4$) ± standard deviation (SD)

† Stylo mulch residues chopped up with a knife in 10 to 15 cm pieces

§ Representative mixture of stylo root DM of four root diameter sizes, i.e., 0.5 to 2.0 mm, 2.0 to 4.0 mm, 4.0 to 10.0 mm and >10.0 mm, cut into 3 to 5 cm long pieces using scissors

¶ Areal density transformed rates of DM and N applied with stylo mulch and root residues in litterbags

to replace non-^{15}N labelled stylo mulch in micro plots, which contained non-^{15}N labelled stylo roots, litter and seeds. The stylo mulch was applied at a rate of 9 Mg DM ha^{-1}. It had a C:N mass ratio of 57 and 7.6 g N kg^{-1} DM, resulting in 6.9 g N m^{-2}, of which 78 mg N kg^{-1} DM was mineral N.

Subsequently, rice amended with ^{15}N labelled stylo mulch was grown. At rice maturity, all rice plants were cut at ground level for yield determination. Rice grains (with hull, i.e., paddy rice) were manually separated from straw with a pedal powered rice thresher. Subsamples of rice grains and straw were dried at 60 °C for 48 h for DM determination. To account for border effects within micro plots, rice plants from the centre were separated from those of the two border rows and discarded after dry weight determination (Dourado-Neto et al. 2010). To calculate rice ^{15}N recoveries from stylo mulch (see below) per micro plot we assumed the same ^{15}N enrichment in rice DM from the border rows and the centre.

Assessment of stylo mulch N recovery in soil N pools

Soil sampling of the 0 cm to 10 cm soil layer was conducted six times during the rice cropping season (Fig. 2). Sampling dates were chosen according to critical rice development stages (Yoshida 1981), i.e., at 5 days before sowing (−5) and +2 (germination), +43 (tillering), +73 (panicle initiation), +93 (late flowering) and +117 (maturity) days after sowing of rice. At rice maturity (+117) soil samples were additionally taken from the 10 cm to 20 cm and 20 cm to 30 cm soil layer. Five soil samples were taken at random locations within each micro plot with a soil auger (ø 1.8 cm, Pürkhauer, Eijkelkamp, Netherlands). The five soil cores from each layer were bulked, homogenised by mixing and a subsample was taken. Subsamples from the topsoil layer (0 cm to 10 cm) designated for soil mineral and microbial biomass N measurements were immediately placed in cooling boxes for transport and subsequently stored on station at 4 °C till further analyses (described below). All other soil samples were dried at ambient temperature and homogenised by sieving (2 mm).

Soil microbial biomass N was measured within one week after sampling using the chloroform fumigation-extraction method (Brookes et al. 1985; Vance et al. 1987). All extracts were collected in 60 ml polypropylene bottles, frozen at −20 °C and shipped to Switzerland. In Switzerland, the samples were unfrozen and the total soluble N in fumigated and non-fumigated extracts was determined using a TOC/TN analyser (Dimatec DIMA-TOC 100, Germany). Microbial N was then estimated from the difference between N extracted from the K_2SO_4 extract of fumigated and non-fumigated soils, expressed as mg N kg^{-1} oven dry soil. No correction factor was used to account for incomplete microbial cell lysis (Joergensen and Mueller 1996). To determine soil mineral N, K_2SO_4 extracts from non-fumigated soil were additionally analysed colorimetrically using a continuous flow injection analyser (SKALAR® San++ System, Netherlands).

^{15}N analyses of microbial and mineral N were conducted in soil sample extracts from +2, +43, +73 and +93 days after sowing rice sowing. The ^{15}N abundance in microbial N was determined by oxidizing fumigated and non-fumigated K_2SO_4 soil extracts (Cabrera and Kissel 1989; Koroleff 1983), followed by the application of the diffusion method of Goerges and Dittert (1998) as modified by Mayer et al. (2003). To measure the ^{15}N abundance in mineral N, extracts from non-

fumigated soil were subjected to the same diffusion method. In brief, during the oxidation step total soluble N in soil extracts is oxidized to NO_3^-, while the principle of the diffusion method is that NH_4^+ and NO_3^- are converted into volatile NH_3, which is then trapped by an acidified filter and analysed for ^{15}N on a mass spectrometer. After drying (72 h), filters were encapsulated in tin-capsules prior to analysis of ^{15}N isotopic composition.

Dried soil and plant sample processing and analyses

From each dried sample a representative subsample was shipped to Switzerland. There plant subsamples were milled with a micro hammer mill (Culatti®, Switzerland) and an ultra-centrifugal mill (Retsch® ZM 200, Germany) to a final size of 0.2 mm. Dried soil samples were milled to a fine powder with a ball mill (Retsch MM 200, Germany. Analyses for total N and C content were conducted on a NCS analyser (Flash EA 1112 series, Thermo Electron Corporation, USA). The ^{15}N isotopic composition was determined on a stable isotope mass spectrometer (Europa Scientific, Crewe, UK) at the Stable Isotope Facilities, Department of Soil Science, University of Saskatchewan, Canada.

Calculations

The abundance of ^{15}N in a compartment expressed in atom% was calculated as

$$^{15}N\ compartment_i = \frac{^{15}N}{(^{15}N + ^{14}N)}\ x\ 100 \quad (1)$$

where i refers to the specific measured compartment.

The natural ^{15}N abundances are expressed as a per mil (‰) deviation from that of atmospheric N_2, i.e., $\delta^{15}N$ (Unkovich et al. 2008):

$$\delta^{15}N\ (‰) = \frac{\text{atom\% } ^{15}N\ compartment_i - \text{atom\% } ^{15}N\ standard}{\text{atom\% } ^{15}N\ standard}\ x\ 1000 \quad (2)$$

where N standard represents the proportion of ^{15}N in the atmosphere (0.3663 atom% ^{15}N).

The %Ndfa for each stylo plant was derived using the average $\delta\ ^{15}N$ of two paired reference plants. The %Ndfa was calculated according to Shearer and Kohl (1986):

$$\%Ndfa = \frac{\delta^{15}N\ reference\ plant - \delta^{15}N\ stylo}{\delta^{15}N\ reference\ plant - B\ value}\ x\ 100 \quad (3)$$

where reference plant refers to the selected non-N_2 fixing reference plant grown on the same soil and the B value is a correction factor for the within-plant fractionation of ^{14}N and ^{15}N between shoots and nodulated roots (Unkovich and Pate 2000). To determine the B-value a pot experiment was conducted (November 2012 to April 2013) in a glasshouse at the research facilities of the radioisotope laboratory (LRI), University of Antananarivo, Madagascar (described in further detail in the supplementary information and Fig. S2). It resulted in a B value of $\delta^{15}N$ -1.19 (SD ± 0.15) ‰, which agreed well with B values published for *Stylosanthes* spp. by Yoneyama et al. (1986) (−0.74, Townsville stylo) and by Nguluu et al. (2001) (−1.60 to −1.86, stylo hamata). When mature rice plants were used as reference plants a weighted mean for the $\delta^{15}N$ was calculated from straw and grain according to Danso et al. (1993). For each plot the average %Ndfa of the five stylo plants was used to calculate the amount of N_2 fixed in stylo shoots from the atmosphere and for statistical analysis. The amount of N_2 fixed in stylo shoots from the atmosphere in kg N ha^{-1} was calculated by multiplying the %Ndfa by the total amount of N (kg N ha^{-1}) contained in the stylo shoot biomass sampled at the end of each cropping season (March). This sampling time corresponded always with the flowering to early seed setting phase of the perennial stylo.

The percentage (PR) of stylo residue DM and its N content remaining in each litterbag were used to calculate the total DM and N release from each litterbag (Cobo et al. 2002);

$$PR\ (\%) = \frac{X_t}{X_0}\ x\ 100 \quad (4)$$

$$Total\ DM/N_{release} = \frac{X_0\ x\ PR}{100} \quad (5)$$

where X_t is the dry weight (in g DM) or N content (in g N) at each sampling time and X_0 the initial dry weight or N content. The N content was calculated by multiplying the remaining DM with its N concentration.

Decomposition rate constants (k) were calculated using regression of $\log_e$ percentage mass remaining on time (t) in days (Anderson and Hetherington 1999);

$$log_e\left(\frac{X_t}{X_0}\right) = -kt \quad (6)$$

Using ^{15}N labelling the proportion of N derived from stylo mulch (%Ndfm) in a compartment was calculated after Hauck and Bremner (1976):

$$\%Ndfm = \frac{AE_{+N}}{AE_M}\ x\ 100 \quad (7)$$

where AE_{+N} is the atom% ^{15}N excess of N in a compartment that received the ^{15}N labelled stylo mulch and AE_M is the atom% ^{15}N excess of N in the stylo mulch. The AE_{+N} was calculated according to Hart and Myrold (1996):

$$AE_{+N} = {}^{15}N\ compartment_i - {}^{15}N\ compartment_{control} \quad (8)$$

where $compartment_{control}$ denotes the natural ^{15}N abundance (atom%) in a soil N pool obtained under identical experimental conditions but without the application of ^{15}N labelled stylo mulch. The AE_{+N} for rice shoots was calculated as weighted AE_{+N} considering grain and straw.

The AE_{+N} of soil microbial biomass N (SMBN) was calculated using a mass balance (Douxchamps et al. 2010; Mayer et al. 2003);

$$AE_{+N}\ SMBN = \frac{\left(TN_F\ x\ AE_{+N(F)}\right) - \left(TN_{NF}\ x\ AE_{+N(NF)}\right)}{\left(TN_F - TN_{NF}\right)}\ x\ 100 \quad (9)$$

where TN_F and TN_{NF} stand for total N concentrations (mg N kg^{-1} soil) measured in extracts from fumigated and non-fumigated soil samples, respectively.

The amount of N in a compartment derived from stylo mulch (Ndfm) was calculated as;

$$Ndfm = \frac{\%Ndfm_{compartment_i}}{100} x\ TN_i \quad (10)$$

where TN is total N in a soil or plant compartment expressed in mg N kg^{-1} soil or g N m^{-2}, respectively. TN was calculated as the product of the concentration of N in the compartment and its weight in g m^{-2} (for plants) or mg kg $soil^{-1}$ (for soil). For soil, the weight of each layer was calculated by multiplying its volume for a 1 m^2 surface by the bulk density. The amount of N derived from the soil in a compartment was calculated by the difference between TN and the Ndfm.

The N recovery in % of added stylo mulch (%MNRec) in a compartment was calculated according to Hauck and Bremner (1976);

$$\%MNRec = \frac{Ndfm_{compartment_i}}{TN_M}\ x\ 100 \quad (11)$$

where TN_M is the amount in g N m^{-2} of applied stylo mulch N.

Statistics

All statistical analyses were carried out using R version 3.0.0 (R-Core-Team 2013). CA treatments (CA-NON, CA-FYM, CA-MIN) were compared using one-way analysis of variance (ANOVA), including blocks as random factor. Post-hoc analyses were done by Fisher's least significant difference test at the $\alpha = 0.05$ level of significance corrected for the number of comparisons

made (Bonferroni correction). Standard errors of the difference in means were calculated from the respective ANOVA. To assess the factor time for labile soil N pools and stylo residue decomposition parameters, sampling dates were compared in a one-way repeated-measures ANOVA using the multilevel approach (i.e., the lme() function and setting the method to ML = maximum likelihood, specifying block and sampling date as random effect). To determine if the mean of the bare fallow MC-NON treatment was significantly different from the mean of the non-amended CA treatment (CA-NON) we performed Welch's two sample t-test. Relationships between variables were determined using Pearson's correlation coefficient. Prior to analysis data was checked for normal distribution using the Shapiro-Wilk test and for homoscedasticity, using and the Levene's test. When assumptions were violated, data was analysed using Wilcox (2012) robust methods (WRS package, version 0.3–1) with trimmed means (0.2).

Results

Stylo above- and belowground biomass production

During establishment as relay crop with rice, stylo growth was slow with little biomass production, in contrast to the fallow season when shoot biomass increased by a factor of up to 20 (Table 2). After four months as relay crop, stylo in the CA-MIN treatment produced 1 Mg DM ha^{-1}, which was twice the amount of shoot biomass produced in the CA-NON treatment. Towards the end of the second rainy season (March 2012) stylo shoot biomass yields were similar for all CA treatments with an average of 10 Mg DM ha^{-1} (Table 2). Total aboveground biomass at the end of the second dry season (October 2012), i.e., when stylo was cut and applied as mulch, was on average 11.4 Mg DM ha^{-1}, of which approximately 25% was fallen stylo litter (Table 2).

Stylo root biomass was much less than aboveground biomass and was mainly concentrated in the top 0 cm to 10 cm. At each sampling (March 2011, 2012), at least 80% of root biomass was recovered in the top 0 cm to 10 cm soil layer (Supplementary information, Fig. S3). Total stylo root biomass in the upper 30 cm of soil was on average 0.5 Mg DM ha^{-1} after four months of stylo growth, which was significantly ($p < 0.01$) lower than the amount of 1.3 Mg DM ha^{-1} retrieved at the second sampling in season two (Table 2).

Stylo above- and belowground dry matter N concentrations

Plant components of stylo differed in their N concentration, which in turn varied over time (Table 2). Stylo shoot N concentration ranged from 8 to 27 g N kg^{-1} DM, and decreased over time. The N concentration in stylo litter sampled after 23 months was on average 18 g N kg^{-1} DM, which was greater than the N concentration in standing stylo shoot biomass at this same sampling date (Table 2). Average N concentration in roots was 10 g N kg^{-1} DM in season one and 13 g N kg^{-1} DM in season two (Table 2).

^{15}N signatures of stylo and reference plants

Irrespective of the cropping season the ^{15}N natural abundance of the reference plants was significantly ($p < 0.05$) higher than that of the respective stylo plant (Supplementary information, Table S1). The average $\delta^{15}N$ of the weedy reference plants was 9.2‰, which was lower than the ^{15}N signature of total soil N (10 to 12‰), but higher than the ^{15}N signature of the rice plants ($\delta^{15}N$ 7.5‰). Stylo grown for four and 17 months had an average $\delta^{15}N$ of 2 and 4‰ (Table 2). The $\delta^{15}N$ of stylo grown as relay crop was on average significantly ($p < 0.001$) lower after four than after 17 months of growth (Table 2). In the second season, there was a residual effect of the fertilizer (applied to rice in season one) on the $\delta^{15}N$ of stylo, with the $\delta^{15}N$ of manure-applied stylo (CA-FYM) being significantly ($p < 0.05$) higher by 1 to 1.5‰ than the $\delta^{15}N$ of stylo grown in the CA-MIN and CA-NON treatment (Table 2).

Stylo N uptake, %Ndfa and N_2 fixation

Stylo shoots accumulated up to 225 kg N ha^{-1} (Fig. 3). The %Ndfa in stylo shoots ranged from 48 to 74%, with an average of 53% towards the end of the second rainy season (Table 2). Total amounts of N_2 fixed in stylo shoot biomass after 17 months ranged from 96 to 122 kg N ha^{-1}, which was more than ten times the amount of N_2 fixed after four months of stylo growth (Fig. 3). The type of fertilizer applied to rice in the first cropping season had a significant ($p < 0.05$) effect on the amount of N_2 fixed in relay cropped stylo grown for four and

Table 2 Dry matter (DM), N concentration, ^{15}N abundance, percent N derived from the atmosphere (%Ndfa) of living and residual stylo plant components sampled after 4, 17 and 23 months of continued growth at the field trial site at Ivory

Season	End of cropping season 1 (March 2011)						End of cropping season 2 (March 2012)						End of dry season 2 (October 2012)				Pre-cropping season 3 (November 2012)	
Months	4						17						23					
Treatment§	Shoot DM Mg ha^{-1}	N g N kg^{-1}	$\delta^{15}N$† ‰	Ndfa## %	Root# DM Mg ha^{-1}	N g N kg^{-1}	Shoot DM Mg ha^{-1}	N g N kg^{-1}	$\delta^{15}N$† ‰	Ndfa## %	Root# DM Mg ha^{-1}	N g N kg^{-1}	Shoot DM Mg ha^{-1}	N g N kg^{-1}	Litter†† DM Mg ha^{-1}	N g N kg^{-1}	Mulch¶ DM Mg ha^{-1}	N¶¶ g N kg^{-1}
CA-NON	0.5	26.2	2.0	65	0.5	9.8	9.8	20.0	3.4	57	1.3	11.8	8.9	7.8	3.0	18.4	9	7.4
CA-FYM	0.6	26.7	1.2	74	0.4	9.7	9.8	20.7	4.9	48	1.2	12.2	8.1	7.9	3.3	18.3	9	6.8
CA-MIN	1.0	27.6	2.4	59	0.6	10.2	10.7	21.1	3.9	54	1.3	14.0	8.3	7.7	2.7	18.5	9	6.8
Mean	0.7	26.9	1.9	66	0.5	9.9	10.1	20.6	4.1	53	1.3	12.7	8.4	7.8	3.0	18.4	9	7.0
SED	0.14	1.76	0.59	6.7	0.14	0.51	0.61	0.78	0.43	4.5	0.27	1.18	1.13	0.45	0.58	0.30	na	0.41
$LSD_{0.05}$§§	0.46	5.77	1.94	22.0	0.46	1.66	2.00	2.60	1.42	14.6	0.88	3.87	3.70	1.47	1.91	1.00	na	1.35
Source of variation																		
Block	ns	ns	ns	ns	ns	*	ns	*	**	*	ns	ns	ns	ns	ns	ns	na	ns
Treatment	*	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	na	ns

Means (n = 4) ± standard deviation (SD); Not applicable (na); significance level *0.05, **0.01, ***0.001; standard error of difference (SED); degrees of freedom: Block = 3, Treatment = 2

§CA = conservation agriculture; no fertilizer (NON), farmyard manure (FYM), NPK + urea (MIN)

§§Fisher`s least significant difference test at the $\alpha = 0.05$ level of significance corrected for the number of comparisons made (Bonferroni)

†$\delta^{15}N$ expressed as a per mil (‰) deviation from that of atmospheric N_2. At the end of the second dry season (23 months) no measurement of stylo $\delta^{15}N$ was conducted

#Roots sampled from 0 cm to 30 soil depth

Percent of total N derived from the fixation of atmospheric N_2, determined using the ^{15}N natural abundance approach

††Litter comprised fallen leaves and inflorescences

¶Stylo shoot residues chopped up with a knife in 10 to 15 cm pieces. Stylo shoot biomass (litter excluded) was adjusted to 9 Mg DM ha^{-1} by removing or adding stylo shoot biomass taken from treatments corresponding to the same cropping history, but not designated for stylo mulch application

¶¶The N concentration was determined from stylo mulch sampled shortly before rice sowing

17 months, which resulted in the following order of treatments: CA-FYM = CA-NON < CA-MIN (Fig. 3). Stylo roots recovered from the upper 30 cm of soil contained an additional 16 kg N ha^{-1} after 17 months of stylo growth. Assuming the same %Ndfa in stylo shoots and roots of approximately 50%, this corresponds to 8 kg N ha^{-1} of fixed N contained in stylo roots.

Residue decomposition and N release

Stylo mulch and root residue decomposition patterns differed, with the highest losses of stylo mulch occurring during the early phase and towards the end of the cropping season, while roots decomposed fastest during the early cropping season (Fig. 4). At the end of the cropping season on average 61% of stylo mulch and 70% of roots were decomposed. Decomposition constants (k) calculated from mass losses were on average higher for stylo roots than for mulch and half-life times were on average 94 (SD ± 40) days for stylo mulch and 72 (SD ± 31) days for roots (Supplementary information, Table S2).

Although residue N release patterns were similar to DM decomposition dynamics, the proportion of N released was higher for stylo roots than mulch throughout the cropping season (Fig. 4). On average 47% of N initially contained in stylo mulch and 72% of N contained in root residues had been released at the end of the experiment. This corresponded to an average daily N release of 15 (SD ± 4) mg N m^{-2} day^{-1} for stylo mulch and 6 (SD ± 1) mg N m^{-2} day^{-1} for root residues (Supplementary information, Fig. S5).

Stylo mulch residue N concentration increased with time, with a simultaneous decrease in the C:N ratio, while root residues showed no significant difference in comparison to their initial composition (Fig. 4). At the end of the cropping season the N concentration in mulch residue DM was 33 (SD ± 8) % higher, while the C:N ratio had decreased from 55 to 36.

Effect of stylo mulch application on soil N pools

Overall soil microbial biomass N ranged from 6 to 20 mg N kg^{-1} soil. For each treatment, microbial N varied little until rice tillering, but tended to decrease from panicle initiation until rice harvest when microbial N was lowest (Supplementary information, Fig. S6). Irrespective of sampling date, the MC-NON treatment had the lowest microbial N, which was on average 8 (SD ± 1) mg N kg^{-1} soil. Across sampling dates the

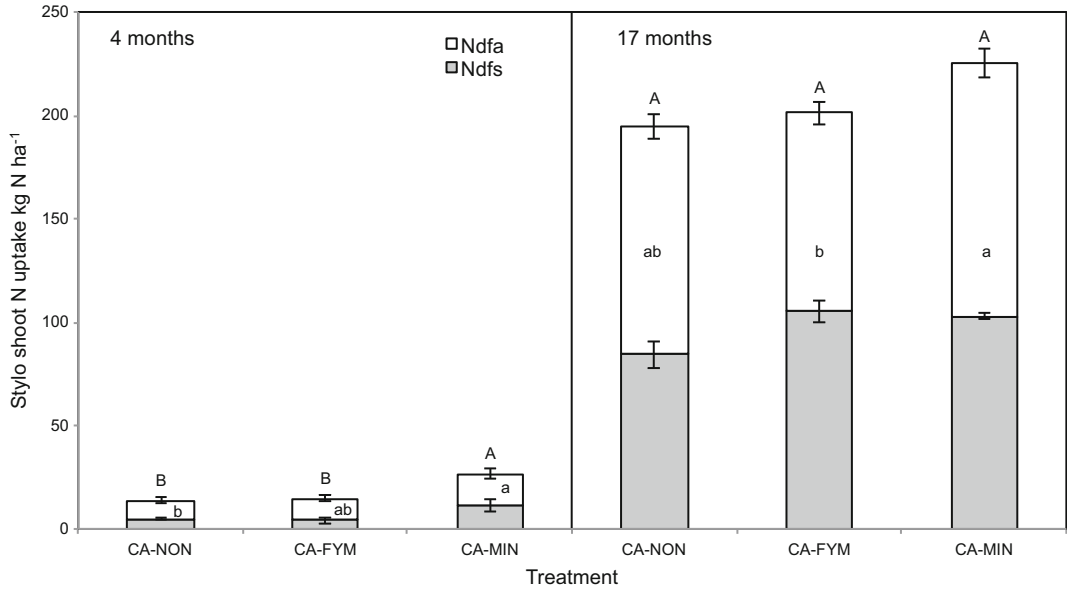

Fig. 3 Stylo shoot N uptake derived from the atmosphere (Ndfa) and the soil (Ndfs) 4 and 17 months after its establishment as relay crop with rice, determined using the ^{15}N natural abundance approach. CA = conservation agriculture; no fertilizer (NON), farmyard manure (FYM) or NPK + urea (MIN). Error bars show ±1 SE (n = 4). Different capital letters on top of stacked bars indicate significant ($p < 0.05$) difference between treatments for total N uptake. Different small letters within stacked bars indicate significant ($p < 0.05$) difference between treatments for Ndfa. For Ndfs there were no significant differences between fertilizer treatments after 4 and 17 months, respectively

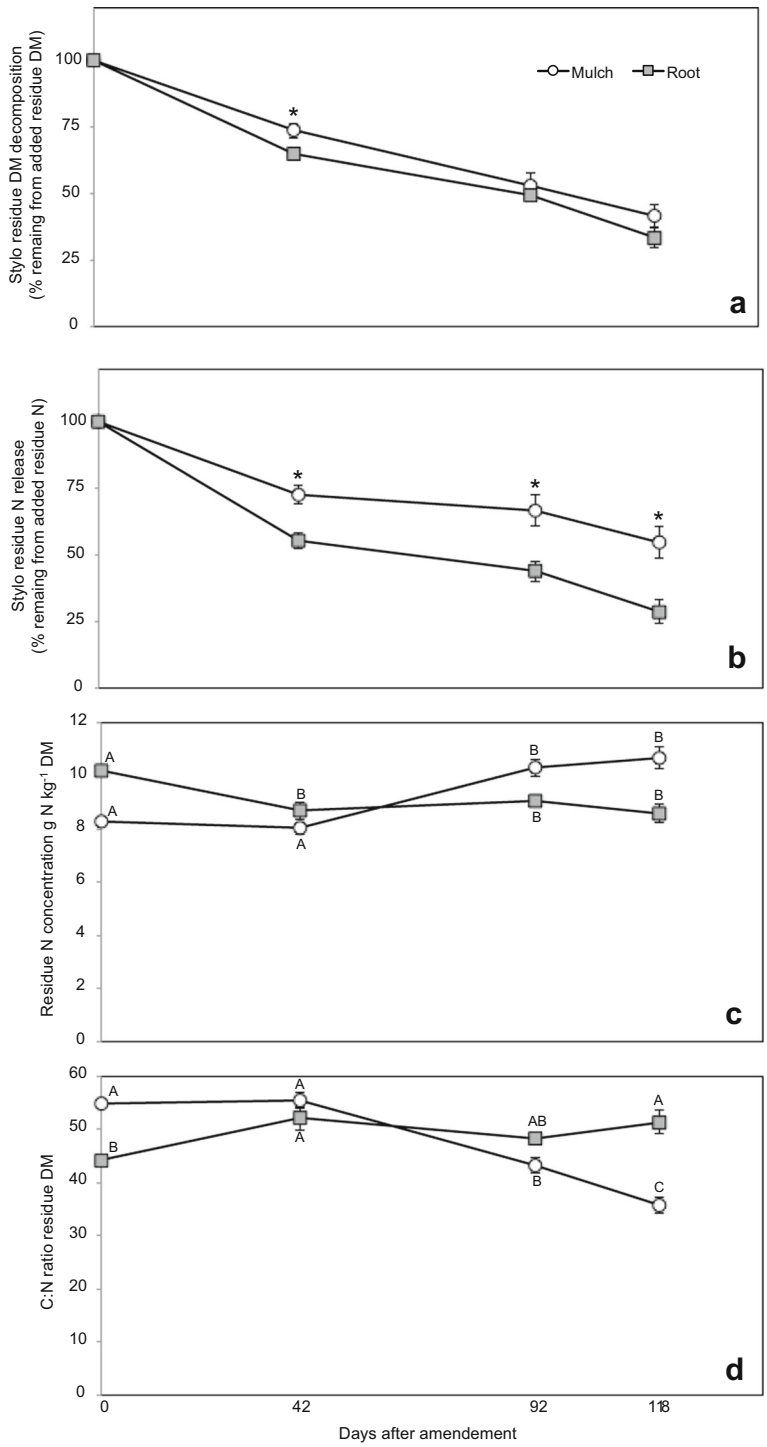

Fig. 4 Dry matter (DM) decomposition (**a**), N release (**b**), N concentration (**c**) and C:N mass ratio (**d**) of stylo mulch and root residues retrieved from litterbags after 0, 42, 92 and 118 days after amendment; Pooled data of all CA treatments are shown because there was no significant effect of fertilizer (applied to rice) treatment. Error bars show ±1 SE ($n = 12$). Asterisks indicate significant ($p < 0.05$) difference between mulch and root residues (a., b.). Different capital letters indicate significant ($p < 0.05$) difference between sampling dates for mulch or roots residues (**c**, **d**)

microbial N was 50% higher in the CA-NON than in the MC-NON treatment.

The proportion of microbial N derived from ^{15}N labelled stylo mulch (%Ndfm) in the CA-NON treatment did not significantly vary during the study and was on average 12 (SD ± 4) % of microbial N (Fig. 5). Corresponding amounts of microbial N derived from stylo mulch averaged 2 (SD ± 0.6) mg N kg^{-1} soil.

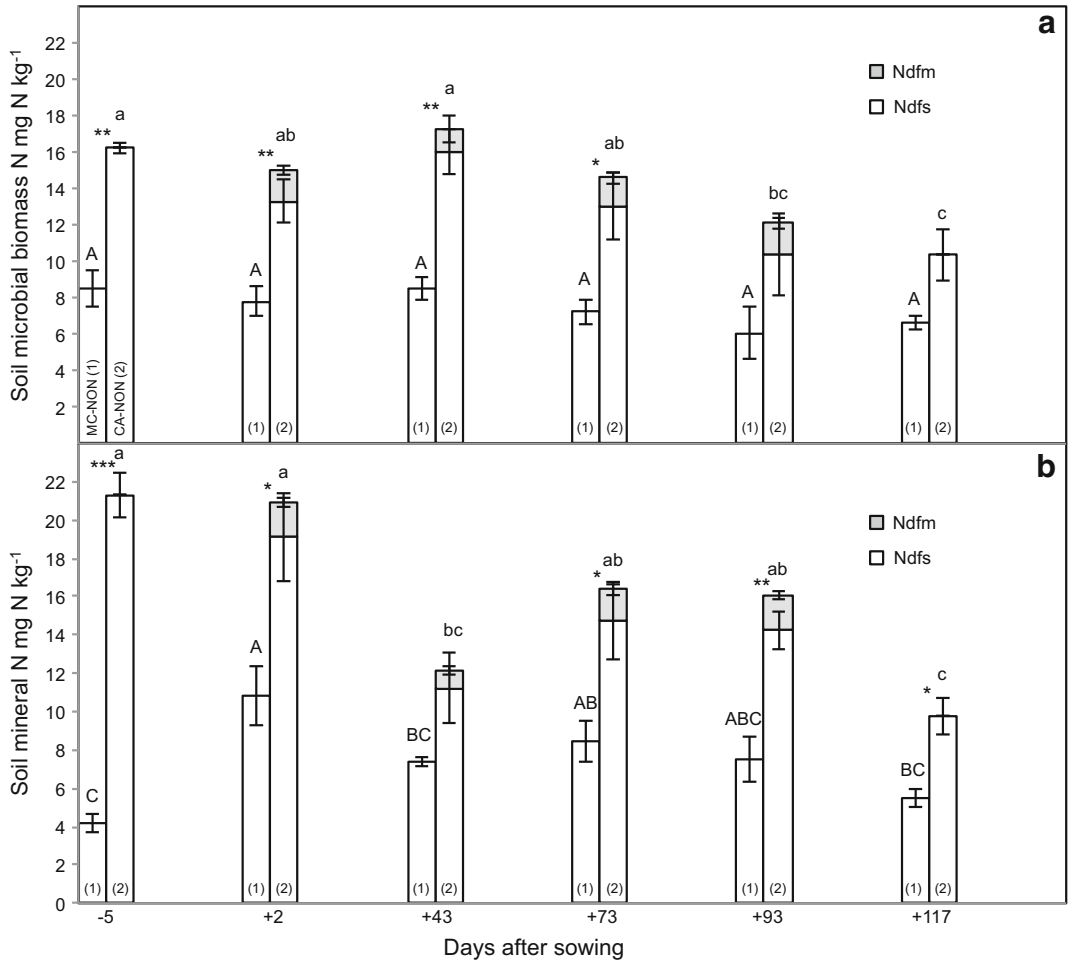

Fig. 5 Soil microbial biomass N (**a**) and mineral (NH_4^+, NO_3^-) N (**b**) in the 0 cm to10 cm soil layer at −5, +2, +43, +73, +93 and +117 days after rice sowing (DAS). Amounts of N derived from soil (Ndfs) and from stylo mulch (Ndfm) were determined at +2, +43, +73 and +93 DAS using ^{15}N labelled stylo mulch. Error bars show ±1 SE ($n = 4$). Asterisks indicate significant (* $p < 0.05$, ** $p < 0.01$) difference of microbial biomass and mineral N between the MC-NON (1) and the CA-NON (2) treatment per sampling date. Different letters indicate significant ($p < 0.05$) difference of microbial biomass or mineral N between sampling dates for the CA-NON (small letters) and MC-NON (capital letters) treatment

On average 3 (SD ± 1) % of ^{15}N labelled stylo mulch were recovered in microbial N.

The soil mineral N pool was overall highest at the start of the cropping season, while it was lowest at rice tillering and at harvest. Mineral N ranged from 4 to 25 mg N kg^{-1} soil, except for the CA-MIN treatment where fertilizer addition shortly before sampling resulted in mineral N values of up to 60 mg N kg^{-1} soil, as expected (Supplementary information, Fig. S6). Throughout the cropping season mineral N was two to five times higher in the CA-NON than in the MC-NON treatment. The %Ndfm in mineral N of the CA-NON varied little during the cropping season and was on average 9 (SD ± 2) %, which corresponded to 2 (SD ± 1) mg N kg^{-1} soil (Fig. 5). The N recovery in mineral N fluctuated between 2 and 3% of applied stylo mulch N.

In bulk soil, the N recovery from ^{15}N labelled stylo mulch was 39%. It was highest in the topsoil (0 cm to 10 cm) and decreased with soil depth (Table 4). At rice harvest total N recoveries from stylo mulch in the soil (0 cm to 30 cm) were similar in all CA treatments with an overall average of 64 (SD ± 13) %.

Rice dry matter yield and N uptake

Grain yield was significantly affected by fertilizer application (Table 3). As expected it was highest (458 g DM m^{-2}) for the CA-MIN and lowest (158 DM m^{-2}) for the

Table 3 Rice grain and straw dry matter (DM) yield, N concentration and uptake harvested from micro plots in cropping season 3 (2012–13)

		Yield		N concentration		N uptake	
		g DM m^{-2}		g N kg^{-1} DM		g N m^{-2}	
Treatment[†]		Grain[††]	Straw	Grain	Straw	Grain	Straw
MC-NON[¶]		158 (77)	124 (48)	12 (0.9)	6 (1.2)	2 (0.8)	1 (0.8)
t - test (p) [¶]		ns	ns	ns	*	ns	ns
CA-NON		271	175	11	4	3	1
CA-FYM		253	159	11	4	3	1
CA-MIN		458	281	14	6	6	2
Mean		327	205	12	5	4	1
SED		36	18	1	1	0.4	0.1
$LSD_{0.05}$[§§]		117	60	2	2	1.7	0.3
Source of variation	df						
Block	3	*	ns	ns	ns	ns	ns
Treatment	2	**	**	**	*	***	***

Means ($n = 4$) ± standard deviation (SD); Not applicable *(na)*; significance level *0.05, **0.01, ***0.001; standard error of difference (SED).

[†] MC = mono cropping, CA = conservation agriculture; no fertilizer (NON), farmyard manure (FYM) or NPK + urea (MIN)

[§§] Fisher's least significant difference test at the $\alpha = 0.05$ level of significance corrected for the number of comparisons made (Bonferroni)

[††] Paddy (grain and husk) yield data were adjusted to the standard moisture content of 0.14 g H_2O g^{-1}

[¶] Results for the MC-NON were not included in the ANOVA. Shown are the mean (± SD) and the t-test probabilities of significant difference to the mean of the CA-0 treatment

MC-NON treatment. The CA-NON treatment produced on average 60% more grain DM than the MC-NON treatment, although this effect was not statistically significant. Except for the CA-MIN treatment, N concentration showed minor variation among treatments and was on average 12 g N kg^{-1} DM for rice grains and 5 g N kg^{-1} DM for straw (Table 3).

Similar to DM yield, mineral fertilizer led to the highest N uptake (8 g N m^{-2}) in rice shoots, with all other treatments ranging between 2.6 and 3.7 g N m^{-2} (Table 3). Although not statistically significant, the amount of N derived from the soil was 30% higher in the CA-NON than in the MC-NON treatment (Fig. 6). The %Ndfm did not significantly differ between CA treatments and was on average 10 (SD ± 3) %, or 0.3 to 0.6 g N m^{-2} (Fig. 6). On average 7% of stylo mulch N was recovered in rice shoots, with overall greater N recovery in grains than in straw (Table 4).

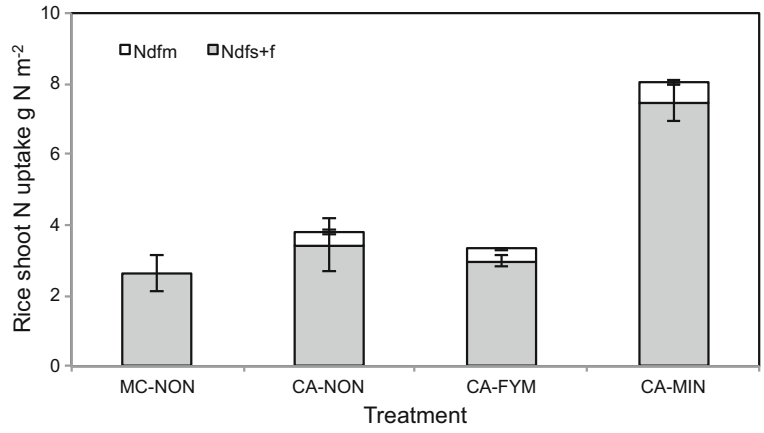

Fig. 6 Rice shoot (grain and straw) N uptake derived from stylo mulch (Ndfm) and from the soil and applied fertiliser (Ndfs + f) determined using ^{15}N labelled stylo shoot residues. MC = mono cropping, CA = conservation agriculture; no fertilizer (NON), farmyard manure (FYM) or NPK + urea (MIN). Error bars show ±1 SE ($n = 4$)

Table 4 ^{15}N recovery from ^{15}N labelled stylo mulch at rice harvest

		^{15}N recovery (% of stylo mulch added at day 0)						
		Surface mulch[§]	Soil			Rice		Total[¶]
Treatment[†]			0-10 cm	10–20 cm	20–30 cm	Grain[#]	Straw	
CA-NON		39	37	20	9	4	1	109
CA-FYM		44	40	18	8	4	1	114
CA-MIN		35	40	12	10	6	2	106
Mean		39	39	16	9	5	2	110
SED		8.4	5.0	3.7	1.5	0.9	0.2	6.6
$LSD_{0.05}$[§§]		27.6	16.5	12.1	4.9	3.0	0.6	21.6
Source of variation	df							
Block	3	ns	ns	ns	*	ns	ns	*
Treatment	2	ns	ns	ns	ns	ns	**	ns

Means ($n = 4$) ± standard deviation (SD); Not applicable *(na)*; significance level *0.05, **0.01, ***0.001; standard error of difference (SED).

[†] CA = conservation agriculture; no fertilizer (NON), farmyard manure (FYM) or NPK + urea (MIN)

[§] Stylo mulch DM remaining at rice harvest in litterbag study; [15] N recovery determined mass balance approach

[#] Rice paddy (grain and husk)

[¶] Total recovery is the sum of recoveries in rice, soil (0 cm to 30 cm) and stylo mulch remaining on the soil surface at rice harvest

[§§] Fisher's least significant difference test at the $\alpha = 0.05$ level of significance corrected for the number of comparisons made (Bonferroni)

Discussion

Stylo N accumulation

The total amount of stylo N accumulation mainly depended on shoot biomass production, since shoot N concentrations and the %Ndfa showed minor variation (Table 2). These results are in line with other studies, e.g., Cadisch et al. (1989) for eight tropical forage legumes including stylo, Ojiem et al. (2007) for stylo and Oberson et al. (2013) for clover. Stylo seems having benefited from the greater N availability in the mineral fertilizer amended rice treatment, as shown in a significantly higher stylo shoot biomass production and shoot N uptake during the first four months of fallow establishment, i.e., in the CA-MIN treatment as compared to the CA-NON and CA-FYM treatment (Table 2, Fig. 3). This suggests that fertilizer inputs to rice during stylo fallow establishment improve stylo growth and N uptake on highly weathered upland soils, as was probably the case for stylo in the CA-MIN treatment.

Somewhat surprisingly, stylo root biomass was low irrespective of treatment (Table 2), which suggests that roots in contrast to shoots (shoot:root DM ratio of 8:1) play a minor part in the total N accumulated by stylo. The low root biomass may explain the importance of mycorrhiza fungi, which are naturally present in many field soils, in the nutrition and growth of stylo, in particular on marginal soils (Jehne 1984). After 17 months, stylo had accumulated on average 16 kg N ha^{-1} in root DM, which was 12 to 15 times less than N taken up in shoot biomass. Our results are supported by the few studies, which assessed stylo root biomass. In a pot study on the effect of soil fertility and NPK application on stylo shoot and root DM production shoot to root ratios ranged from 3:1 (-NPK) and 9:1 (+NPK) (Tian and Kang 1998). Szott et al. (1994) quantified root biomass of stylo to a soil depth of 45 cm in managed fallow systems on a tropical Ultisol (Acrisol) in Peru with an annual rainfall of 2200 mm. At 17, 29, 41, and 53 months after fallow initiation root biomass irrespective of aboveground biomass (8 to 18 Mg ha^{-1}) was less than 5 Mg ha^{-1}. In a study on the effects of P and K supply on growth and N_2 fixation of tropical forage-legumes cultivated in an Oxisol of the Eastern Plains of Colombia Cadisch et al. (1989) determined less than

5 kg N ha^{-1} in stylo root biomass recovered from 0 cm to 20 cm soil depth.

The physical recovery of roots however, provides only a rough estimate of stylo root N and DM accumulation, since the method does not consider fine roots or seasonal rhizodeposition. In a review, Fustec et al. (2010) reported that legume rhizodeposition (decomposition and decay of nodules and root cells and exudation of soluble N compounds by plant roots) may comprise 4 to 71% of total plant N. In a meta-analysis of published data on N_2 fixation in Australian crop and pasture legumes (Unkovich et al. 2010) calculated 'root factors' of 2.0 (*Medicago sativa)* and 1.7 (*Trifolium subterraneum*) to multiply shoot total N by to estimate shoot + root N. Using such a factor for the conversion of aboveground legume N to estimate total N accumulation would result in significantly higher N contribution of stylo roots. In our study, soil mineral and microbial N after stylo fallow were substantially greater in the CA-NON treatment than in rice mono cropped (MC-NON) soil. However, only a small proportion of the surplus in soil mineral and microbial N was derived from the ^{15}N labelled stylo mulch (Fig. 5). This indicates that residual N of non-^{15}N labelled stylo root N contributed to these pools over the following rice season, although we cannot entirely separate that effect from the N effect of non-^{15}N labelled aboveground litter and seed fall during the dry season 2012 (see below).

Seasonal stylo residue N turnover and stylo mulch N recovery in soil N pools

Residue decomposition and N release patterns can mostly be explained by climatic conditions, although in the case of this study also the contact of the residues with the soil need to be considered (i.e., stylo mulch applied on the soil surface, root residues in soil). The high early residue N release (Fig. 4) is in line with other studies looking at residue decomposition in tropical agro ecosystems. For example, Thomas and Asakawa (1993) observed during the rainy season a release of 60% of stylo litter N after 14 days and 80% after 42 days. Such early N release and residue decomposition dynamics can be explained by the onset of the rains and resulting dry–wet cycles, which enhance physical breakdown of residues and promote microbial residue decomposition (Birch 1964).

Fungi may also have played a major role in stylo mulch turnover. At rice harvest about 70% from root DM and N had been lost from the litterbags, while the DM loss (61%) from stylo mulch was higher than the proportion of N (47%) released (Fig. 4). Thus, N loss from stylo mulch was slower than loss of DM, resulting in increasing N concentrations in mulch residues with time (Fig. 4). The same phenomenon was also observed by Urquiaga et al. (1998), Salas et al. (2003), Hart et al. (1993), Frey et al. (2000) and Matos et al. (2011), who related the increase in N concentration to the decomposition of residues to fungal rather than bacterial decomposition. Fungi maintain a number of characteristics that present advantages over bacteria to decompose low quality residues placed on the soil surface (Rasche and Cadisch 2013). Most importantly, their extensive hyphae network allows fungi to utilize both the surface residue C and soil N and moisture (Beare et al. 1992; Holland and Coleman 1987; Yuste et al. 2011). Further, as hyphal growth proceeds, nutrients are immobilized in the hyphal cell walls where their potential for mineralization is low (Kassim et al. 1981). This indicates that the increase in N concentrations in stylo mulch residues at the later stages of decomposition could be due to immobilized N within stylo mulch residues related to colonisation by fungi, although a net N release occurred. Visual observations made at litterbag recovery that showed extensive fungal growth on shoot and root residues (Supplementary information, Fig. S7), support this assumption.

Overall, most of ^{15}N labelled stylo mulch N (~ 64%) was recovered in the top 30 cm of bulk soil (Table 4). This high N recovery in the soil was consistent with other studies on organic residues in tropical agro-ecosystems (Dourado-Neto et al. 2010; Douxchamps et al. 2011) and indicates a build up of potentially mineralizable N in soil. However, in our study only 61% of the stylo mulch had been decomposed at rice harvest (Fig. 4), with the non-decomposed remainder, corresponding to 39% of ^{15}N applied with stylo mulch, still on the soil (Table 4). We assume that this remainder contributes upon decomposition to the N input in soil from stylo mulch, though the extent may depend on the removal by soil macro fauna (Vanlauwe et al. 1998) and the occurrence of bushfires during the dry season (Kull 2016).

The fate of stylo residue N not recovered in the litterbags is not entirely clear. At rice harvest, most of ^{15}N labelled stylo mulch N was recovered in the top 10 cm of soil (Table 4). However, little of this was present in the soil microbial and mineral N pools, in which together around 6% of stylo mulch ^{15}N was

recovered. Likewise, only about 1% was recovered in the dissolved organic N pool (calculated as the difference between total soluble N (extracted with 0.5 M K_2SO_4) and soil mineral N (van Kessel et al. 2009) Supplementary information, Fig. S8). Therefore, we assume that most of the stylo mulch N transferred to the topsoil layer, but not recovered in specific soil N pools remained in the pool of non-decomposed residues, i.e., physically uncomplexed organic matter (light fraction; specific gravity 1.6 to 2.0 g cm^{-3}) and particulate organic matter (> 53 to 2000 mm in diameter)) (Beare and Gregorich 2007), which may contain 13–40% of total soil N (Haynes 2005). Further research would be needed to characterize its form in order to evaluate its potential and temporal availability as N source for subsequent crops and the build up of soil N stocks. As discussed below, the total mulch N recovery suggests that little mulch N was lost from the fields.

Stylo mulch N uptake and recovery by rice

The immediate and long-term N benefit from legumes will depend largely on the proportion of legume derived N taken up by the succeeding crop and its recovery in the soil. Using ^{15}N labelled stylo mulch showed that the %Ndfm in rice (grain and straw) was similarly low (~9%) in all treatments, irrespective of fertilizer application, with most N derived from the soil and fertilizer (Fig. 6). The corresponding ^{15}N recoveries in rice of 5 to 8% from stylo mulch (Table 4) were similar to N recoveries in maize (8.5%) from applied ^{15}N–labelled legume (*Leucaena leucocephala*) residues in alley cropping systems in Nigeria (Vanlauwe et al. 1998) and in maize (7.3%) from applied ^{15}N–labelled legume (*Crotalaria juncea*) residues in the Guinea savannah zone in northern Ghana (Fosu et al. 2003). In our study, the low stylo mulch N recovery in rice reflects the low mineralization of stylo mulch N as a result of stylo mulch placement on the soil and its C:N ratio of 55. However, despite the low N supply by stylo mulch to rice, stylo culture increased rice N uptake by an additional 30%, as shown in the difference in the amount of N derived from the soil in rice between the CA-NON and the MC-NON treatment (Fig. 6). Most likely, this reflects the greater (on average 75%) seasonal soil mineral N in the CA-NON than in the MC-NON treatment (Fig. 5) and in part explains the 70% increase in rice grain yield in the CA-NON treatment (Table 3). This may be because of stylo N contribution beyond that by ^{15}N labelled stylo mulch, which is discussed below.

Total ^{15}N recoveries slightly greater than 100% at rice harvest, in the remaining surface mulch, rice and soil down to 30 cm (Table 4), suggest that N losses from the system were likely to be minor. This could be linked to the stabilization of stylo mulch N derived particulate organic matter through biochemical stabilization and physical protection within soil aggregates (Six et al. 2002), leading to lower rates of mineralization and losses via leaching, which is particularly important under tropical rainfall conditions. The reason of total ^{15}N recoveries greater than 100% of the total ^{15}N added with stylo mulch may lay in the susceptibility of the ^{15}N tracer technique to changes in soil bulk density although in this study outmost care was taken to accurately determine the soil bulk density in the different treatments.

Known and potential stylo N pools and flows

Stylo N related pool sizes changed over time. The following combination of measured and estimated stylo N pools and flows (Fig. 7) broadens our understanding of stylo N supply and identifies future research questions. At the end of the second cropping season (March 2012, 17 months of stylo growth) stylo had accumulated 195 to 225 kg N ha^{-1} in shoot biomass (Fig. 3), suggesting a high potential N source for the following rice crop. However, only half of this N was found at the final harvest after the dry season (October 2012, 23 months of growth), with on average 65 and 55 kg N ha^{-1} in stylo shoots and litter (Table 2). Clearly, the fate of N contained in stylo litter demands for further investigation, since also other authors (Becker and Johnson 1998; Muhr et al. 2002; Saito et al. 2010) did not pay attention to fallen leaves as potential crop N source. The lack of about 100 kg N ha^{-1} from stylo biomass N after the dry season could be explained by plant internal translocation of accumulated N from vegetative to regenerative plant components (seeds) during the post-cropping season (Gardener et al. 1982; Vallis and Gardener 1984) and to drought tolerance and avoidance mechanisms that allow the plant to survive the long dry season (Fisher and Ludlow 1984; Gardener 1984; Little et al. 1984; Mott et al. 1981; Probert 1984). Indeed, stylo produces a high number of small seeds (230,000 to 1,250,000 seeds ha^{-1}), which depending on the species can amount to a total of 620 to 1763 kg ha^{-1} (Hopkinson and Walker 1984). For the species *Stylosanthes guianensis*, 827 and

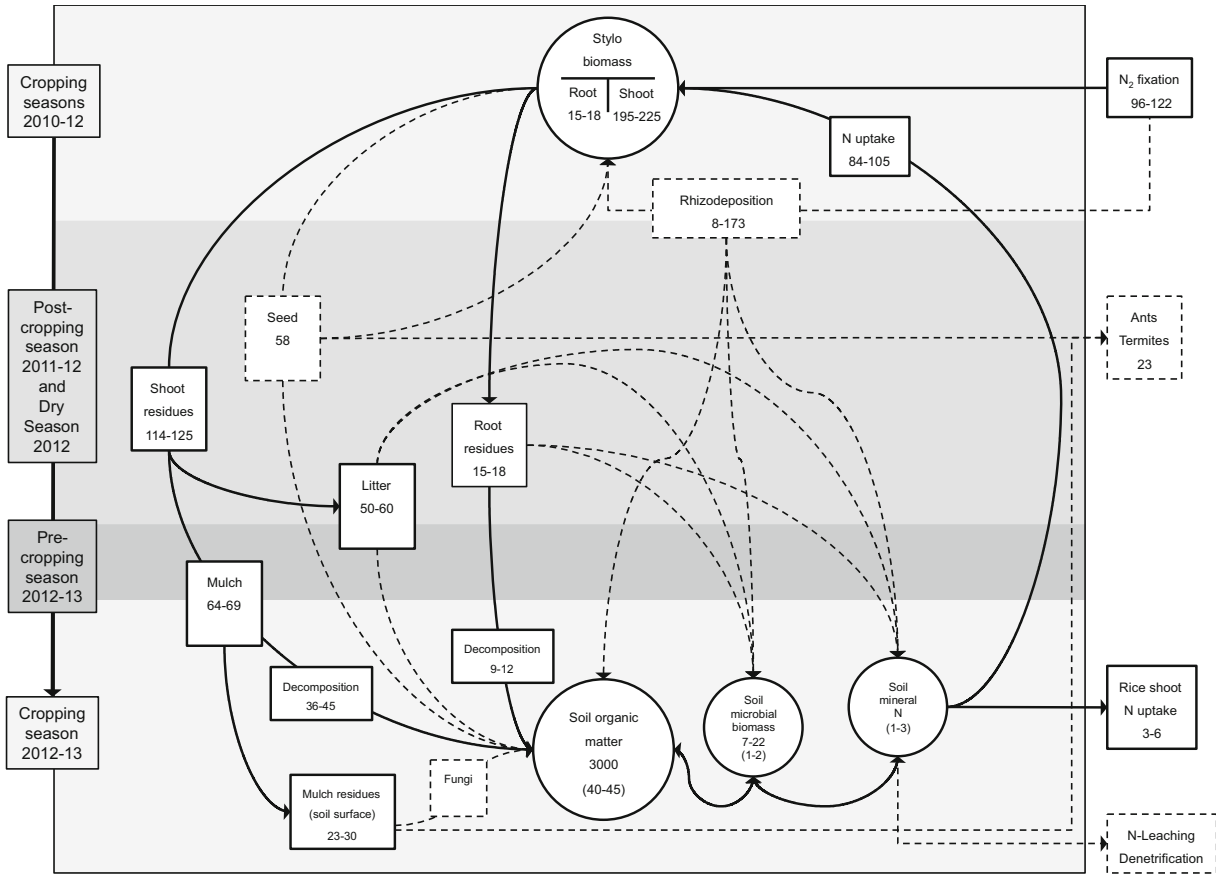

Fig. 7 The distribution and cycling of N accumulated by *Stylosanthes guianensis* (stylo) as a fallow crop in a three-year conservation agriculture based crop rotation with upland rice on a Ferralsol in the Midwest Highlands of Madagascar. Solid lines and solid rectangles show quantified N fluxes and distribution of total stylo biomass N into sub-pools. Dashed lines and dashed rectangles show estimated N fluxes and distributions. Circles represent the main N pools; Quantities of fluxes, distributions, and pools are in kg N ha^{-1}; Figures in brackets show the amount of stylo N recovered in the respective N pool. Shape sizes do not correspond to N amounts. Gray scale in the background shows time. Rhizodeposition N estimate (range 4 to 71% of total plant N) are based on Fustec et al. (2010)

1000 kg ha^{-1} have been reported by Gardener (1984). Assuming a seed N concentration of 58 g kg^{-1} DM (determined in seeds of this study's B-value experiment), which is at the lower end of stylo seed N concentrations from 56 to 68 g kg^{-1} DM reported by Gardener et al. (1982), and a seed N production of 1000 kg ha^{-1}, suggests that 60% of the N not found in aboveground stylo biomass after the dry season could be related to seed formation. A large proportion of these seeds can rest in the soil (deLeeuw et al. 1992; McKeon and Mott 1984; Orr 2010). An evaluation of tropical pasture species as leys in the semi-arid tropics of northern Australia showed that seeds of stylo can establish a seed bank in the soil for over 4 years (Cameron 1996). Potential losses, for example of up to 40% of N removed by ants and termites were reported by McKeon and Mott (1984).

Whether the other missing 40% of N not found in aboveground stylo biomass after the dry season have been transferred to the root systems remains unclear, since roots were not sampled after the dry season. However, it seems realistic as shown in a pot study under drought conditions reported by Vallis and Gardener (1984), where the proportion of total plant N transferred into the roots can range from 13 to 34% of total plant N during flowering and seed setting. Thus, this translocation could have increased the potential amount of 17 kg N ha^{-1} stored in root residues (only considering N concentration and amounts of DM determined in April 2012, Table 2) and its N release upon decomposition during the following rice season. As discussed above, the physical recovery of roots however neglects fine roots and seasonal rhizodeposition. Using allocation factors to

relate legume shoot N to rhizodeposition (Fustec et al. 2010; McNeill and Fillery 2008; Unkovich et al. 2010) would result in a significantly higher N contribution of stylo roots.

In sum, latter N fluxes and pools derived from N accumulated in stylo biomass after 17 months may explain why unexpected stylo mulch in contrast to other stylo N components (seeds, litter, roots) played a limited role for rice yield through its N supply. To properly understand short term yield increases through improved crop N supply other stylo N components (seeds, litter, roots) need to be studied in greater detail (using ^{15}N labelling). Finally, since CA is considered a long term approach, there is also a need to study the effect of successive crop rotational cycles with stylo mulch application on crop N supply and yield through the build up of soil N stocks, as indicated by the high total N recoveries from stylo mulch remaining on the soil surface and in the soil at rice harvest.

Conclusions

This study has shown that increasing crop N supply and yield is possible through integration of legumes such as stylo in low input CA upland cropping systems on highly weathered tropical soils. Under the edaphic conditions of the Malagasy highlands stylo accumulated irrespective of fertilizer input substantial amounts of N in its aerial biomass, of which about half was derived from N_2 fixation. Yet, the contribution of the assumed major stylo N source, i.e., stylo mulch, to the following rice crop was small because its decomposition was incomplete and N released was mainly present in non-plant available form. The ^{15}N labelled stylo mulch did not represent the total stylo N input, but only about 30% of stylo N present at 17 months of stylo growth. Such, stylo N released from litter and seeds fallen off during the dry season and from roots seems to be an additional important N source, which should be further investigated and be included in predictions on stylo N supply to crops. Higher mineral and microbial N pools after stylo fallow as compared to the rice mono crop treatment indicated that stylo litter, seeds and root residues increased the soil mineralization potential beyond the effect detected with the ^{15}N labelled stylo mulch. High total N recoveries from stylo mulch remaining on the soil surface and in the soil can be important for the build up of soil organic N stocks, which are crucial to improve crop N supply and soil fertility in low input upland cropping systems on highly weathered tropical soils.

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Authors' contributions OZ: Conception and design, fieldwork, laboratory analysis, data interpretation and analysis, manuscript writing; EF, AO, and ES: Conception and design, coordination, data interpretation and manuscript revision. All authors read and approved the final draft.

Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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