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RESEARCH ARTICLE

Biological nitrogen fixation by legume cover plants in oil palm plantations: calibration of the ureide technique and effects of plantation age and soil nitrate

Rachel Pipai · Ann McNeill · Murray Unkovich · Murom Banabas · Paul N. Nelson

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Abstract

Background and Aims To sustainably manage N in oil palm systems quantities of N fixed by cover legumes need to be understood. Current values are scarce, based on shoot N measures and do not include litter which releases nitrate as it decomposes. We aimed to quantify N_2 fixed by legumes under oil palm systems in PNG and to determine if soil nitrate influenced dependence on N_2 fixation (Ndfa).

Methods The ureide technique for estimating tropical legume Ndfa was calibrated for *Calapogonium mucunoides and Pueraria phaseoloides* using ^{15}N isotope dilution, and then used to assess Ndfa for legume cover under oil palms (2 to 25 years old) in Papua New Guinea. Amounts of fixed N in aboveground legume biomass (shoot plus litter) were calculated incorporating % groundcover. Soil nitrate under the legume litter was also measured.

Results Legume Ndfa was highly negatively correlated with soil nitrate concentration but independent of palm age. Legume groundcover, shoot and litter dry matter, and quantity of fixed N were greater under oil palms less than 5 years old, decreasing under older plantations where solely *C. caeruleum* was present. DM and N content of litter were similar to shoots for legumes in plantations less than 6 years old.

Conclusion The calibrated ureide technique can be used, together with estimates of annual legume N accumulation, to quantify N input from legume groundcover during the life cycle of oil palm plantations and other tropical ecosystems, in order to support more sustainable management of N.

Keywords Calapogonium · Pueraria · Mucuna · Standing biomass · Litter · Nodulation

Introduction

Oil palm (*Elaeis guineensis*) has become one of the world's major agricultural crops, driven by unprecedented increase in consumer demand (Corley 2009) coupled with rapid commercial development, with production occurring in lowland moist humid tropical zones in Central Africa, South America and Southeast Asia (Byerlee et al. 2016). Although 85% of global production currently comes from Indonesia

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R. Pipai · A. McNeill (✉) · M. Unkovich
School of Agriculture Food & Wine, Faculty of Sciences, Engineering and Technology, The University of Adelaide, Waite Campus, Urrbrae, SA 5064, Australia
e-mail: ann.mcneill@adelaide.edu.au

R. Pipai · M. Banabas
Oil Palm Research Association, Kimbe, Papua New Guinea

P. N. Nelson
College of Science and Engineering, James Cook University, Cairns, Australia

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and Malaysia, the oil palm industry is a highly important component of the export economy for many other countries, including Papua New Guinea (PNG), where it is the largest non-government employer and underpins rural livelihoods (Allen et al. 2009; Cramb and Curry 2012). There is increasing pressure from consumers and governments for agriculture and food production systems to be more sustainable, with oil palm under particular public scrutiny to improve productivity per current area rather than encroach further on important natural environments (Teoh 2010; Sayer et al. 2012). Many palm oil producing countries, including PNG, are committed to increase the sustainability of the oil palm industry via activities undertaken as members of the Roundtable on Sustainable Palm Oil (RSPO, https://rspo.org/as-an-organisation/).

A key factor for sustainability of any agricultural system is the management of nitrogen (N) in order to maximise productivity and minimise off-site effects, particularly eutrophication of waterways caused by run off and nitrate (NO_3^-) leaching, and greenhouse gas (GHG) emissions from denitrification and volatilisation (Galloway et al. 2013). Nitrogen fertilizers, a significant component of the cultivation costs in oil palm systems (Corley and Tinker 2016), are considered a major potential source for such N losses (Choo et al. 2011; Comte et al. 2012). Amounts of mineral fertiliser N applied to oil palm plantations vary widely, ranging from 48 to 90 kg N ha/year for immature palms to 56–206 kg N/ha/yr for mature palms (see references cited in Pardon et al. 2016). Whilst there is a dearth of comprehensive data to describe the complete N cycle of an oil palm system from planting to harvest, modelling using the limited data available suggests potential for N losses is greatest during the immature phase (first 2–3 years) when N uptake by palms is low, and then later during the mature phase when groundcover under palms tends to be sparse and N fertiliser rates higher (Pardon et al. 2016). The most influential parameters for N loss from an oil palm system were identified as mineral N fertiliser rate, drainage and fraction of legume in groundcover (Pardon et al. 2017).

Perennial legume species such as *Pueraria phaseoloides, Calapogonium mucunoides Calopogonium caeruleum, and Mucuna pruriens* have long been utilised as groundcover for control of soil erosion and suppression of weeds under oil palm plantations (Fairhurst and Hardter 2003; Samedani et al. 2015), as well as for N input via biological N_2 fixation or BNF (Giller and Fairhurst 2003). Amounts of N fixed by cover legumes, including *P. phaseoloides* under 2–3 year old oil palm in Malaysia, were estimated at 150 kg N/ha/yr (Agamuthu and Broughton 1985) based on the mean difference in N content between the vegetation of the N_2 fixing legume and non-fixing "natural" covers (N difference method). Another Malaysian oil palm study using ^{15}N isotope-dilution methodology reported similar amounts of N_2 fixation by *P. phaseoloides* (Zaharah et al. 1986). However, published measures of BNF for cover legumes in oil palm systems in situ remain scant (Giller and Fairhurst 2003; Pardon et al. 2016), despite a number of methodologies available for field use (Unkovich et al. 2008).

One approach for assessing the dependence of a legume cover on BNF that does not require the presence of a non-fixing cover is the ureide technique (Herridge 1982). This method is based on the premise that many tropical legume plants, from the tribes *Phaseoleae* and *Desmoideae* within the subfamily *Papilionoideae*, transport identifiable N_2 fixation products in the xylem sap as ureides in contrast to N from mineral N assimilated into amides (Peoples et al. 2009). While the method itself is inexpensive and relatively simple, it must be calibrated with another technique such as the natural abundance $\delta^{15}N$ ($‰$) or ^{15}N isotope dilution technique (Unkovich et al. 2008). Ureide calibrations have been carried out for a number of legume species, including *Desmodium ovalifolium* and *Centrosema spp.* that are commonly used as oil palm covers. Stem relative ureide N (RUN) for these species has been shown to be highly correlated with the proportion of N derived from N_2 fixation (pNdfa) assessed using ^{15}N isotope dilution, proving the reliability of the calibrated ureide technique (Unkovich et al. 2008). Although there is mention of an unpublished ureide calibration for *C. caeruleum* (Wilson et al. 1995), there appear to be no published calibrations for this species, or for *C. mucunoides*, *P. phaseoloides* and *M. pruriens* (Unkovich et al. 2008) which are widely used as cover legumes in oil palm systems.

As plantations age and the palm canopy closes, the legume cover often declines due to shading, although some species thrive under older plantations (Giller and Wilson 1991). Reduced legume cover growth means lower amounts of N_2 fixed

(Zaharah et al. 1986), but it is unclear how plantation age and accompanying increase in shade, affects the proportional dependence on N_2 fixation of the legumes. Also, since pNdfa tends to be reduced if nitrate is present in the legume root zone (Streeter and Wong 1988), it might be expected that accumulation of nitrate in soil, resulting from increased accumulation and decomposition of cover legume litter as plantations age, would reduce legume dependence on N_2 fixation. However, a glasshouse study to determine the effect of litter quantity on pNdfa for *P. phaseoloides* suggested that effects were small as it was only reduced from 87 to 84% when litter input was increased from zero to the equivalent of 8 Mg DM/ha (Vesterager et al. 1995). Legume covers in tropical environments accrue litter rapidly within 6–9 months of establishment (Broughton 1977; Chiu 2004; Samedani et al. 2015), but reports of the rate at which this litter decomposes vary widely from a few months up to a year or more (Vesterager et al. 1995; Turner and Gillbanks 2003; Chiu 2004; Philip and Abraham 2009). Furthermore, although incubation experiments with ^{15}N labelled residues of the cover legumes *P. phaseoloides* and *M. bracteata* demonstrate rapid net N mineralisation (Mendham et al. 2004), it has been suggested the flux may be reduced in the plantation situation by N immobilisation due to the presence of other residues from living and previously felled trees in the plantation (Pardon et al. 2016). It has been reported that the presence of cover legumes actually reduced leaching of N in plantations (Agamuthu and Broughton 1985; Lehmann et al. 1999) but there appears to be no direct investigation of soil nitrate under cover legumes in the field specifically related to BNF inputs in oil palm systems.

Therefore, this study aimed to quantify N_2 fixation by legume cover crops under oil palm systems in PNG and to determine possible relationships between pNdfa and amount of nitrate in the soil under the cover legumes. The ureide technique was calibrated for legume cover species in the glasshouse, subsequently used to estimate pNdfa of legume covers in the field under different ages of palm, and then amounts of fixed N in total aboveground biomass (shoot plus litter) of legume covers were calculated.

Materials and methods

1. Glasshouse experiment to calibrate the xylem ureide technique using ^{15}N isotope dilution

Plant culture and growing conditions

A glasshouse experiment was conducted to measure changes in the relative ureide abundance in stems of nodulated legumes grown at varying nitrate supply, with N_2 fixation measured using ^{15}N isotope dilution. The experiment took place under natural daylight over 9 months (May-January) at the Waite campus of the University of Adelaide, South Australia (34.9688° S, 138.6339° E). Average day/night temperature in the glasshouse for the growth period was 25/17°C. During winter (June - August) supplementary glasshouse lighting was used to ensure a daylight period of 12 h. Seeds of *C. mucunoides*, *P. phaseoloides* and *M. pruriens* were sourced from the Australian Tropical Grains Germplasm Centre (Queensland). Prior to sowing *C. mucunoides* seeds were soaked in hot (75°C) water for three minutes to break dormancy (Giller and Fairhurst 2003) and *P. phaseoloides* seeds were soaked for one hour in glycerol at 50°C. *M. pruriens* seeds required no pre-treatment. Single seeds of each species were sown in free-draining 4-L pots containing fine acid-washed river sand in May (*C. mucunoides* and *M. pruriens*) and July 2011 (*P. phaseoloides*). All pots were inoculated with Group M *Bradyrhizobium* strain (CB756, Becker Underwood, Australia), by mixing peat inoculum in water and pouring a small amount of slurry into each seeding hole before the seeds were sown.

Nutrient solution and nitrate treatments

The glasshouse experiment had five nitrate (NO_3^-) concentration treatments including zero, four replicates and two harvests for each species, giving a total of 120 pots, arranged in a randomized complete block design. Pots were watered as needed with tap water until the first true leaves were observed on the plants, and until 10 weeks of age fed with a basal minus N nutrient solution for tropical legume culture (Herridge and Peoples 2002b) containing (as g/L) 0.0625 $MgSO_4$, 0.0368 $CaCl_2$, 0.02188 KH_2PO_4, 0.0188 KCl, and 0.0259 FeEDTA and as (mg/L)

0.715 H_3BO_4, 0.4525 $MnCl_2$, 0.0275 $ZnCl_2$, 0.0125 $CuCl_2$ and 0.0063 $NaMoO_4$, initially diluted 1/8 or ¼ according to the growth rate of the seedlings. Nitrate feeding of plants commenced ten weeks after sowing using ^{15}N labelled potassium nitrate (5.0 atom% ^{15}N) mixed with unlabelled $Ca(NO_3)_2.4H_2O$ and unlabelled KNO_3 to make 1, 3, 5 and 10 mM NO_3^- solutions. Resulting enrichments of these nitrate solutions were 1 (1mM) 0.33 (3 mM), 0.2 (5mM) and 0.1 (10mM) atom % ^{15}N excess in the above nutrient solutions. Pots were fed 1 L of nutrient solution twice weekly until harvest.

Sampling

Plants were harvested on different dates due to different growth rates, with *M. pruriens* harvested 16 and 21, *C. mucunoides* 29 and 34 and *P. phaseoloides* 23 and 28 weeks after sowing. At harvest, plants were cut just above the sand surface and 7 stem pieces (12–15 cm long) excised and oven dried at 85°C for 48 h and weighed, as was the remainder of the shoot. Each pot was tipped over carefully to remove loose sand and ensure the root system plus nodules remained as intact as possible. Roots were washed clean using tap water and a 2-mm sieve used to capture the remaining roots and nodules from the wash bucket. Each intact root system was divided into 0–5 cm, and >5 cm sections in relation to depth in the sand. Nodules were separated from the root and counted, before roots and nodules were oven-dried at 85°C for 48 h and weighed. All dried plant samples were ground in a ball mill.

Analyses of plant material

Dried samples of legume shoots, roots and nodules were analysed for total N and ^{15}N using an automated combustion analyser linked to a mass spectrometer (Dawson and Brooks 2001). Ground stem samples were subject to solute extraction and the extracts stored in a freezer prior to analysis for ureide, amino and nitrate-N as described in Unkovich et al. (2008). Briefly, ureide-N was analysed using the Rimini-Schryver reaction (Young and Conway 1942), amino compounds were analysed using the ninhydrin method (Yemm and Cocking 1955) and nitrate was analysed using the salicylic acid method (Cataldo et al. 1975).

Calculations

The concentration of ureides in the stem solutes relative to the amino and nitrate N concentrations, which derive principally from soil nitrate uptake (Herridge and Peoples 2002a), can be expressed as relative ureide N (RUN). Given that there are four N atoms in the ureide molecule, the RUN (%) can then be represented as:

$$\text{RUN}\ (\%) = (4a/(4a + b + c)) \times 100$$

where *a* is the concentration of ureides; *b* nitrate-N and *c* amino-N (Herridge 1982).

The relationship between RUN (%) and the proportion of plant N derived from the atmosphere (pNdfa, %) has been shown to be close to unity for a range of legumes (Unkovich et al. 2008).

In the present study the pNdfa (%) was calculated using the ^{15}N isotope dilution technique with the ^{15}N analysis data and the following equation (Unkovich et al. 2008).

$$\text{pNdfa}\ (\%) = [1 - (\text{atom}\ \%\ ^{15}\text{N excess in plant}/\text{atom}\ \%\ ^{15}\text{N excess in nutrient solution})] \times 100$$

2. Field survey of N_2 fixation by legumes under oil palm

Site information

Field studies were carried out in thirteen plantations across three commercial oil palm estates (Kapiura, Mosa and Numundo) near Kimbe (5.5512° S, 150.1387° E), West New Britain Province, PNG. The climate in the region is tropical with rain every month and an average annual rainfall of 3600 mm. Oil palm ages in the 13 sampled plantations ranged from 2 to 25 years with each plantation approximately 27 ha. Fertiliser application in the year prior to sampling (2011) varied with age of palm and management approach (Table 1). Although a mix of legume cover species had generally been sown immediately after the palms were planted as per best practice recommendation (Fairhurst and Hardter 2003) there were only three plantations, two on the Kapiura estate and one on the Mosa estate, where more than one species (i.e. *C. caeruleum* with *P. phaseoloides*) contributed

Table 1 Palm age (years), legume cover species present, soil pH at sampling and fertiliser applied (kg/palm) in the year 2011 prior to the sampling in Jan-Feb 2012, for plantations sampled on three commercial oil palm estates near Kimbe, West New Britain Province PNG.

Estate	Palm Age (yrs)	Legume Cover Species	Soil pH $_{CaCl2}$	Fertilizers applied in 2011 (kg/palm)				
				N	P	K	Mg	B
Kapiura								
	3	*C. caeruleum*	4.3	0.73	0.11	1.05	0.12	0.01
		P. phaseoloides	4.4					
	5	*C. caeruleum*	4.9	0.87	0.12	1.44	0	0
		P. phaseoloides	5.2					
	7	*C. caeruleum*	4.6	0.48	0	0	0	0
	25	*C. caeruleum*	4.7	0	0	0	0	0
Mosa								
	2	*P. phaseoloides*	4.4	0.89	0.19	1.01	0.09	0.02
	4	*C. caeruleum*	5.1	1.05	0.11	1.42	0.14	0
		P. phaseoloides	5					
	6	*M. pruriens*	5.6	1.04	0.18	1.25	0.14	0.01
	13	*C. caeruleum*	5	1.04	0.18	1.45	0.14	0.01
	18	*C. caeruleum*	4.8	1.37	0.25	1.45	0.09	0
Numundo*								
	4	*P. phaseoloides*	nd	1.01	0	1.45	0	0.01
	8	*C. caeruleum*	nd	0.82	0	0.83	0.09	0.01
	9	*C. caeruleum*	nd	0.65	0	0	0	0.01
	16	*C. caeruleum*	nd	0.24	0	0.33	0	0

nd = not determined

* Sampling in Numundo estate was restricted to legume cover and stem segments for solute analysis whereas locations in the other two estates were also sampled for shoot and litter dry matter

to the ground cover (Table 1), whereas at all other plantations only one species was present. (Table 1). The soils are volcanic ash in origin (Banabas et al. 2008). Sampled soils pH_{CaCl2} ranged from 4.3 to 5.6 (Table 1) and were sandy loam to clay texture in the top 30 cm.

Soil and plant sampling and analyses

Soil and plant sampling was conducted in January and February 2012. In Kapiura and Mosa estates, for each legume cover species present, ten replicate samples of above-ground biomass (green shoots and litter separately) within $1m^2$ quadrats were taken at each plantation. Quadrats were placed randomly in areas containing the specific legume cover. Stem segments (10 cm length) were also obtained from each quadrat. Sampling at the Numundo estate plantations was restricted to stem segments and assessment of legume ground cover. Shoot, stem and litter samples were oven dried (85°C), weighed, ground, subsampled and transported to Australia for analysis of total N and N solutes extracted from stem segments as described for the glasshouse study. Soil was sampled to a depth of 20 cm using an auger (15 cm diameter) in four of the quadrats at each plantation. The four samples were thoroughly mixed, subsampled (~500 g) and oven-dried at 60°C. Sub-samples of the dried soils (~100 g) were sent to Australia. To comply with Australian quarantine regulations all dried plant and soil samples were gamma irradiated at a dosage of 25kGray (Steritech Pty Ltd, Queensland), prior to release for analysis. Sub-samples of each soil were then extracted with 2 M KCl (40ml/10 g soil) for one hour and the extracts analysed on an Alpkem Flow Solution II Analyser for nitrate-N and ammonium-N using colorimetry (Rayment and Lyons 2011). Further sub-samples were extracted in 0.01 M $CaCl_2$ (1:5) for one hour and after allowing sediment to settle for 30 min, pH in the supernatant was measured with a Thermo ORION 960 pH/conductivity electrode (Rayment and Lyons 2011).

Estimation of legume ground cover (%) and amount of fixed N in standing shoot biomass

Ground cover (%) for each legume species present in a plantation was assessed using a representative

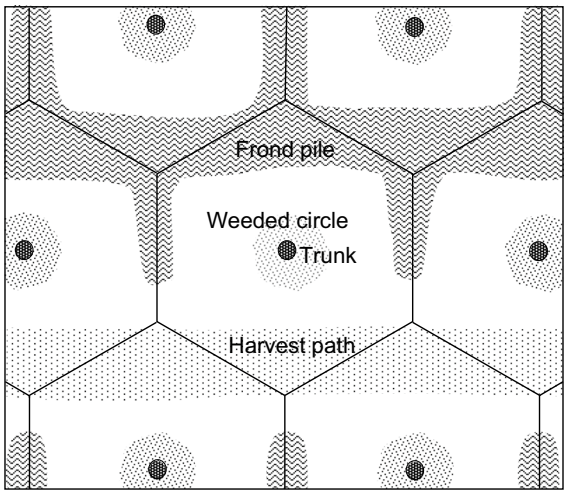

Fig. 1 Hexagonal 'palm unit' assessed for % legume species cover at each location. Palm spacing varied with planting density but at all locations was close to the standard triangular distance between adjacent palms of 9×9×9 m (Corley and Tinker 2008). The weeded circle diameter is 1 m radius at time of palm planting and is gradually expanded to 2 m radius by palm age 2 years

hexagonal 'palm unit' (Fig. 1) that accounted for the triangular layout of palms, a weeded circle, the cleared harvest path and the frond piles. A quadrat ($1m^2$) was moved from place to place within the hexagonal area to assess the total area (m^2) covered by the legume species. Cover was observed to grow across harvest paths and on top of frond piles but the weeded circle usually remained as bare soil. The amount of N fixed by the legumes in standing shoot biomass in the plantation was calculated as the product of estimated % legume cover, pNdfa (%) as determined from stem ureide analysis and application of the calibration equation generated in the glasshouse study, and measured N contents of the shoots. To calculate the total amount of above-ground fixed N (in standing biomass plus litter) a simple assumption was made that the pNdfa (%) measured for the shoots was also applicable to the N in the litter under those shoots.

Legume nodulation assessment

Legume nodulation was visually assessed on randomly selected plants at all plantations on the Mosa and Kapiura estates using a scoring system based on number and position of nodules on the excavated root system, modified from Corbin et al. (1977). Categories were poor (score 0–2); fair (2–3), good (3–4) and excellent (5) nodulation, with the lowest considered indicative of little or no N_2 fixation and the highest implying that N_2 fixation potential is near optimal.

Statistical analysis of data

Analysis of variance was used to explore differences in species responses to NO_3^- supply in the glasshouse experiment. Linear regressions were established between RUN (%) and pNdfa (%) from the glasshouse experiment. Firstly a general linear homogeneity of slopes analysis was used to determine if there were differences between species in the slopes of the regressions (MANCOVA, $P<0.05$). If there were no differences between species then a single linear regression was derived using the mean species data. All analyses were conducted with the Statistica™ software package (v13.5.0.17, TIBCO Software Inc., CA USA). For the field data, general relationships between variables were explored using visual lines of best fit (power curves) using Microsoft Excel. The field experiments were based on a need to gather quantitative data on N_2 fixation and as such were descriptive rather than hypothesis driven.

Results

1. Glasshouse experiment to calibrate the relative ureide N technique for cover legumes

Proportional dependence on nitrogen fixation (pNdfa, %) measured by ^{15}N isotope dilution

Since the only source of N in the zero NO_3^- treatments was N_2 fixation, these plants are assumed to have fixed 100% of their N. The NO_3^- supply regime was effective in providing a wide range of dependencies on N_2 fixation (Fig. 2A). Across the two harvests pNdfa (%) decreased with increasing N supply, especially for *C. mucunoides* and *P. phaseoloides* (Fig. 2A) which appeared very similar in their response, with 5mM NO_3^- being sufficient to suppress N_2 fixation by approximately 90%. In contrast, *M. pruriens* maintained a significantly ($P<0.01$) higher dependence on N_2 fixation compared with the other two species under all NO_3^- additions.

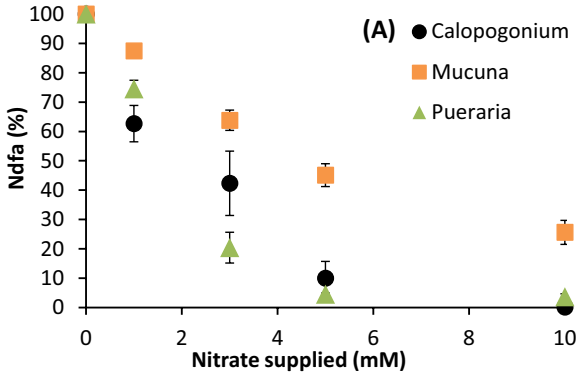

Fig. 2 **A** Legume dependence on N_2 fixation (Ndfa, %) as a function of NO_3^- supplied in the glasshouse, and **B** nodule mass (g/plant) as a function of NO_3^- supply for *C. mucunoides*, *P. phaseoloides* and *M. pruriens*. Values are means across two harvests. Error bars are standard error of the mean for n = 8

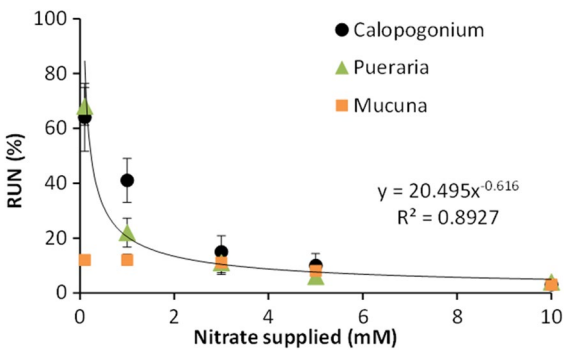

Fig. 3 Relative stem ureide N (RUN, %) for (*C mucunoides*, *P. phaseoloides* and *M pruriens* as a function of NO_3^- supplied in the glasshouse. Values are means across two harvests. Error bars are standard error of the mean for n = 8. A single (power) curve has been fitted across data for *C mucunoides* and *P. phaseoloides*

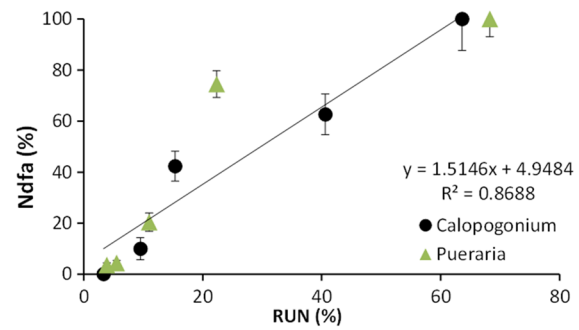

Fig. 4 Correlation of Ndfa (%), derived using ^{15} N isotope dilution technique, and stem RUN (%) for *C. mucunoides* and *P. phaseoloides*. Data are means from two sampling times in the glasshouse experiment. Error bars are standard error of the mean for n = 8

Nodule mass per plant (Fig. 2B) was much greater for *M. pruriens* than the other two species, and as for N_2 fixation, was suppressed less than for the others under increasing NO_3^- supply. Nodule number (data not shown) also significantly decreased with increasing nitrate supply in *P. phaseoloides*, but not in the other two species.

Stem ureide N calibration

Stem RUN (%) indices were very similar in the two harvests (data not shown) and thus mean values across harvests within a species and NO_3^- supply are presented (Fig. 3). Mean RUN was the same ($P < 0.01$) for *C. mucunoides*, and *P. phaseoloides* at each rate of NO_3^- supply (Fig. 3A), except for the 1 mM treatment, in which *C. mucunoides* had a higher RUN (40%, $P < 0.01$) than the other two species. The RUN for *M. pruriens* for the zero and 1mM nitrate treatments was much lower than for the other species (Fig. 3A) and similar to values for the higher nitrate treatments, suggesting ureide production in this species is not necessarily related to nitrogen fixation.

Plotting the ^{15} N-estimated Ndfa (%) against the measured RUN (%) for each NO_3^- concentration again showed the similarities between *C mucunoides and P. phaseoloides* (Fig. 4). Homogeneity of slopes analysis showed that the regressions for *C mucunoides and P. phaseoloides* were not

significantly different from each other ($P > 0.05$ by analysis of variance), and thus they were combined to produce a single calibration curve relating Ndfa (%) to stem RUN (%) for use in the field study (viz. Ndfa (%) = 1.514 x RUN (%) + 4.9848). While the correlation between Ndfa and stem RUN for *M. pruriens* (data not shown) was no weaker than for the other two species ($R^2 = 0.8763$), the slope was very much steeper (Ndfa (%) = 7.4857 x RUN (%) – 2.5601), meaning that an absolute change of 1% in stem RUN for *M. pruriens* resulted in a shift in Ndfa of 7.5% with the comparable shift for the other species of 1.5%. This further indicated that RUN is unlikely to be a useful indicator of pNdfa for *M pruriens*.

2. Field Survey of legume cover species in oil palm plantations

Legume cover shoot and litter dry matter and N content

Shoot dry matter for total legume cover sampled in each plantation ranged from 144 g/m^2 to 792 g/m^2 (mean 347 g/m^2) across all plantations (Fig. 5A). Shoot biomass was greater in plantations with young oil palm, especially where cover was a mixture of two species (*P phaseoloides and C caeruleum*), and less in plantations with older (7–25 year) palms where cover was mainly *C. caeruleum*. As a result, there was a strong correlation ($R^2 = 0.67$) between legume cover shoot dry matter and age of palm across plantations (Fig. 5A), with shoot biomass decreasing rapidly in the first 5–10 years, after which it remained at around 150 g/m^2. Litter collected directly under the legume shoots averaged 762 g/m^2 in plantations 6 years old or less and 139 g/m^2 for those with palms 7–25 years

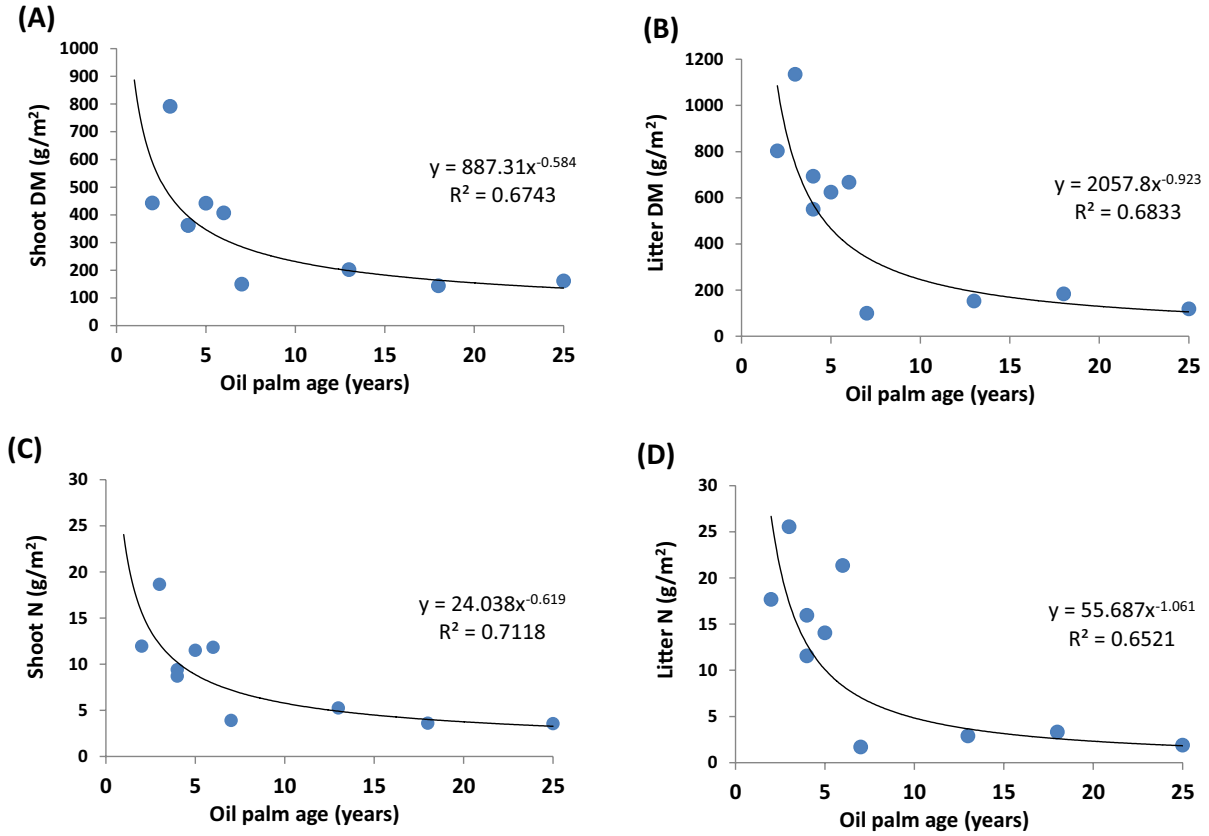

Fig. 5 Correlations between age of oil palm plantation and **A** shoot or **B** litter dry matter (DM), and **C** shoot or **D** litter N content for total legume cover sampled from different aged palm plantations in West New Britain Province, PNG. Power curves have been fitted between age of oil palm and total legume cover DM or N per plantation

old. The litter mass was almost always greater than the standing shoot biomass, regardless of palm age or legume species present, and was also strongly correlated with oil palm plantation age (Fig. 5B).

Shoot N concentration for legumes ranged from 2.2% (*C. caeruleum*, Kapiura 25 year old plantation) to 2.9% (*M. pruriens*, Mosa 6 year old). There were no significant differences ($P = 0.44$) between shoot N concentrations of *C. caeruleum* and *P. phaseoloides*, however species differences remain unclear because *P. phaseoloides* was only found in young palm plantations and there was a strong negative correlation (Adj $R^2 = 0.64$, $P < 0.01$) between the age of the stand and the shoot N concentration. The litter N concentrations for individual species (1.6 to 3.2%) were marginally lower than those of the associated legume shoots, and more markedly so in older plantations. Legume shoot N content was 3.6 (*C. caeruleum*, Kapiura, 25 year old) to 18.6 (*P. phaseoloides*, Mosa 2 year old) g/m^2 and legume litter N content was 2.0 (*C. caeruleum*, Kapiura, 25 year old) to 21.8 (*M pruriens*, Mosa 6 year old) g/m^2. There were good correlations between legume shoot N content and palm age (Fig. 5C, $R^2 = 0.71$) and litter N content and palm age (Fig. 5D, $R^2 = 0.65$), as reported for dry matter.

Stem RUN

C. caeruleum generally had higher stem RUN than *P. phaseoloides*, ranging from 17 to 43% (average 28%), while the range for *P. phaseoloides* was 13 to 38% (average 18%). Furthermore, in the glasshouse study RUN in *C. mucunoides* obtained at zero N and 10mM N were very similar to those reported for fully nodulated (80% RUN) and fully nitrate-dependent (10% RUN) *C. caeruleum* (Gault and Peoples 1993), suggesting the two species of that genera are very similar in ureide activity. This gave confidence for applying the calibration derived for RUN in *C. mucunoides* to the *C. caeruleum* cover present in the field. RUN for the single *M. pruriens* sampled in the field survey was 28%, exceeding the maximum (12%) recorded for that species in the glasshouse experiment.

Legume proportional dependence on N_2 fixation (pNdfa, %) for cover species

Mean pNdfa was significantly higher ($P < 0.05$) for *C. caeruleum* (49%) than for *P. phaseoloides* (32%), although for both species a wide range of values were observed (Table 2). There was no correlation between plantation age and pNdfa for *P. phaseoloides* or *C. caeruleum* (Fig. 6A) although *P. phaseoloides*, which was only present in younger plantations, generally had low pNdfa (14–27%) and consistently appeared to be deriving a larger proportion of its N from soil (72–82%) than *C. caeruleum* (25–72%, Table 2).

Soil extractable N

Concentrations of nitrate-N in the soil extracts ranged from 2 to 25 mg NO_3^--N/kg. The highest NO_3^--N occurred in soil from under *P. phaseoloides* in a 3 year old plantation on the Kapiura estate and the lowest NO_3^--N in soil under *C. caeruleum* from a 25 year old plantation on the same estate. There was a strong negative relationship ($R^2 = 0.55$) between soil nitrate concentration and pNdfa (%) of individual cover legumes (Fig. 6B), with a marked decline in dependence on N_2 fixation as nitrate increased above 5 mg NO_3^--N/kg.

There were exceptionally high concentrations of ammonium-N (95–292 mg NH_4^+-N/kg) in all the soil extracts and a poor correlation between pNdfa (%) and ammonium-N concentration in soil ($R^2 = 0.02$). It was suspected that ammonium concentrations in the soils may have been influenced by the irradiation treatment of the samples on import to Australia.

Nodulation score

A high nodulation score (4 or 5) indicating an excellent potential for N_2 fixation was recorded for both *P. phaseoloides* and *C. caeruleum* in plantations that were 5 years old or less (Table 2). A nodulation score of 4 was also recorded for *C. caeruleum* in a 13 year old Mosa plantation, whereas in the majority of plantations with older palms the nodulation scores for all three legumes were 2–3 indicating fair nodulation but reduced N_2 fixation potential. A nodule score of 1 was assigned to *C. caeruleum* in the 18 year old Kapiura plantation suggesting little or no N_2 fixation activity by this cover under mature trees. However, there were no obvious correlations between nodule score and either soil nitrate-N or pNdfa (%) of individual legume species (Table 2).

Table 2 Groundcover (%), proportional dependence on N_2 fixation (pNdfa, %)[1], nodulation (Nod) score[2] and DM, total N and N derived from N_2 fixation (kg/ha) for above ground biomass (standing shoot plus litter) of the legume cover species present under oil palm plantations of different ages in West New Britain Province, PNG. Numbers in bold are for the total legume present at each plantation.

Estate								Above-ground legume biomass (kg/ha)						
Plantation ID	Palm Age (yrs)	Legume Species	% ground cover	s.d.[3]	pNdfa (%)	s.d.	Nod score	DM	s.d.	Total N	s.d.	Fixed N	s.d.	Fixed N as % Tot N
Kapiura														
K3	3	*C. caeruleum*	9	2	39	13	5	779	416	18	10	7	5	
		P. phaseoloides	8	3	18	7	5	787	423	18	9	4	2	
		Total	**17**					**1566**		**36**		**11**		**31**
K5	5	*C. caeruleum*	19	3	75	60	2	625	206	15	10	12	10	
		P. phaseoloides	10	5	28	16	4	610	201	15	5	4	2	
		Total	**29**					**1235**		**30**		**16**		**53**
K7	7	*C. caeruleum*	**7**	6	37	15	2	**148**	108	**3**	< 1	**1**	< 1	**33**
K25	25	*C. caeruleum*	**< 1**	1	53	28	3	**16**	26	**< 1**		**< 1**	< 1	nd
Mosa														
M2	2	*P. phaseoloides*	**44**	8	22	7	3	**5593**	2020	**135**	52	**34**	8	**25**
M4	4	*C. caeruleum*	20	13	51	16	4	1986	1761	43	10	25	6	
		P. phaseoloides	2	3	26	19	3	233	301	5	7	2	4	
		Total	**22**					**2219**		**48**		**27**		**56**
M6	6	*M. pruriens*	**5**	5	nd		2	**670**		**21**	32	**nd**		**nd**
M13	13	*C. caeruleum*	**5**	6	28	19	4	**172**	193	**4**	2	**2**	3	**50**
M18	18	*C. caeruleum*	**10**	16	55	28	1	**475**	840	**9**	10	**6**	10	**67**

[1] Ndfa (%) derived from measured ureides in solute extracts from field stems using a ^{15}N isotope dilution calibration (Unkovich et al. 2008)

[2] Nodulation score based on a modified Corbin et al. 1977; [3] s.d.Standard deviation nd not determined

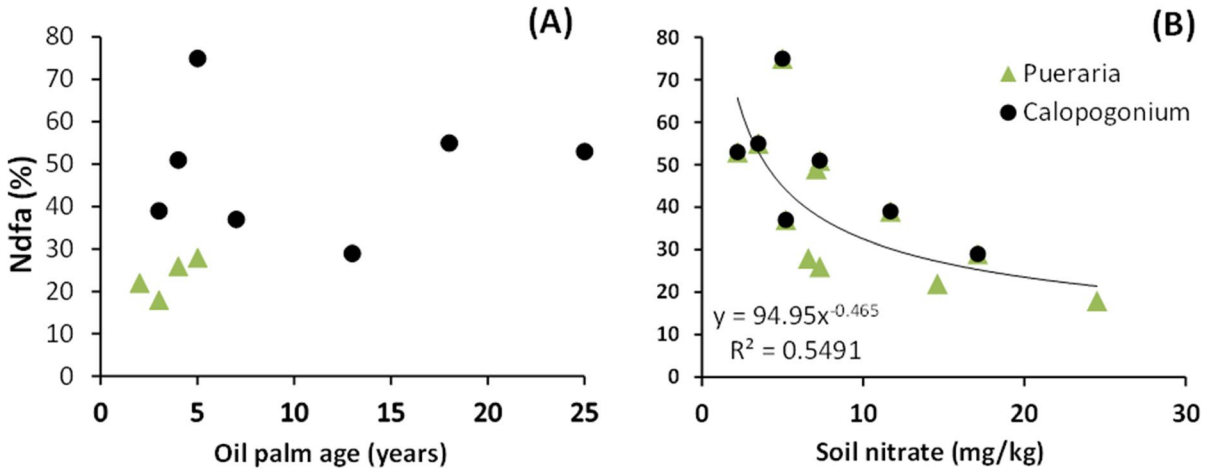

Fig. 6 Scatterplots showing legume dependence on N_2 fixation (Ndfa,%) in relation to **A** oil palm age or **B** nitrate-N in 0-20 cm soil depth under cover legumes (*C. caeruleum*, and *P. phaseoloides*) in different aged oil palm plantations in West New Britain Province, PNG.

Plantation legume ground cover (%) and total standing biomass (DM), N and fixed N (kg/ha)

The proportion of ground covered by legumes in different plantations was highly variable and ranged from 0.6% in the oldest plantation sampled (*C. caeruleum*, Kapiura 25 year old) to 44% (*P. phaseoloides*, Mosa 2 year old). There were insufficient samples in the survey (Table 2) to be able to ascertain if there were trends in species presence with age of oil palm plantation, particularly as *M. pruriens* was only encountered in one plantation. However, it was evident from the restricted dataset (Table 2) that greater legume ground cover was recorded in plantations that were 5 years old or less, and that only *C. caeruleum* was present under the older plantations. Generally the percentage legume ground cover decreased as oil palm age increased ($R^2 = 0.67$).

Assuming legume ground cover (%) across the entire plantation was similar to that in the sampled regions then the total biomass of above-ground legume cover (standing shoot plus litter) could be 1.2–5.6 Mg DM/ha containing 30–135 kg N/ha in plantations five years old or less (Table 2). This includes three plantations where mixtures of cover species occurred. Whereas in plantations older than five years standing shoot plus litter biomass (16–670 kg DM/ha) and N (< 1-21 kg N/ha) would be markedly lower, corresponding with observations of less cover and lower shoot/litter N concentrations in these plantations.

The amount of fixed N in shoots plus litter ranged from 2 to 34 kg N/ha (Table 2) but was not correlated with plantation age. Nevertheless, amounts of fixed N in total legume above-ground biomass (sum of standing shoot plus litter of all species present) were generally higher in the younger plantations where dry matter was greater due to more legume cover, and in some cases two cover species present (Table 2). Fixed N in total legume above-ground biomass was partitioned between standing shoots and litter in a ratio of 1:1 or 1:>1 in plantations less than 6 years old and in older plantations (with one exception) at a ratio of 1:1 or 1:<1. The proportion of N in total legume above-ground biomass derived from N_2 fixation was greater than 30% for 6 of 9 plantations across two estates (Table 2), partly due to relatively high pNdfa for *C. caeruleum* (Table 2).

Discussion

A calibrated ureide technique was used, for the first time globally as far as we are aware, to estimate proportional dependence on N_2 fixation (pNdfa) by cover legumes in oil palm plantations. Stem RUN for *P. phaseoloides* and *C. caeruleum* in a field survey in PNG were within the range (11–76%) reported for these species from a Malaysian rubber plantation

study (Norhayati et al. 1988). Dependence on N_2 fixation by the legume covers in this study ranged from 18% (*P. phaseoloides*) to 75% (*C. caeruleum*) and was highly variable between and within species, although overall *C. caeruleum* exhibited higher pNdfa than the other species. The pNdfa obtained for *P. phaseoloides* at 4 sites in this field survey in PNG were generally also lower than reported for this species in other tropical field studies using ^{15} N-based methods (Cadisch et al. 1989; Sanginga et al. 1996a, b; Giller 2001b; Fosu 2003; Sanginga 2003). The glasshouse study provided a robust calibration of the ureide technique (using ^{15}N isotope dilution) for *C. mucunoides* and *P. phaseoloides*, and individual calibrations for *C. mucunoides* and *P. phaseoloides* were similar to those of pigeonpea (Peoples et al. 1989) and common bean (Unkovich et al. 2008). The limited range of ureide production in the glasshouse study for *M. pruriens* was similar to that observed for the tree legume species *Gliricidia sepium* which is considered a non-ureide exporter with respect to nitrogen fixation (Herridge et al. 1996). Indeed, it is possible that the colorimetric assay (Young and Conway 1942) used for analysing ureide in stem extracts of *M pruriens* in this present study could have given false positive results due to interference by non-ureide compounds, as has been shown for xylem sap extracts of *Sesbania grandiflora* (Herridge et al. 1996). The potential occurrence of this could be determined in future by checking the absorbance spectra of the final coloured products of the assay (Peoples et al. 1991). Such false data would render the apparent strong linear relationship between pNdfa and RUN for M pruriens invalid. Furthermore, the higher RUN value obtained for the single sample of *M. pruriens* in the field may have been related to time of sampling since significant diurnal fluctuation in ureide production has been reported, even for legumes where ureide production is not linked to pNdfa (Herridge et al. 1996). Overall we conclude that the RUN technique may not be suitable for estimating pNdfa in *M pruriens* Nevertheless, the ureide calibrations for the other two species from this study do provide an avenue to more widely use this reliable and fairly simple technique in future N_2 fixation studies of legume covers in oil palm plantations or other agroecosystems.

It was apparent from the field survey that pNdfa for legume covers did not show any relationship with age of oil palm plantation, and hence age of these perennial legume covers. However, as one might expect given evidence in the literature (cited in the introduction) and data from the glasshouse study derived using ^{15}N isotope dilution, pNdfa was negatively correlated with NO_3^- concentration in the soil under the cover legumes. Across all species and ages of plantation pNdfa was significantly lower where soil NO_3^- -N concentrations were above 5 mg/kg. Data from the glasshouse study also indicates that N_2 fixation in *C. mucunoides* and *P. phaseoloides* was very sensitive to low concentrations of available NO_3^-. *M pruriens* on the other hand appears less sensitive and may continue to fix N_2 under conditions of higher available N in the root zone. Thus, where mineral N in the soil is high, *C. mucunoides* and *P. phaseoloides* are likely to be more dependent on uptake of NO_3^- from soil than *M. pruriens* and this may decrease the potential for loss of N by leaching or through denitrification under these two species. Studies of *P. phaseoloides* as a cover crop with fruit trees in an Amazonian rubber plantation, using ^{15}N-labelled fertiliser, similarly concluded that it was a good scavenger of applied N (Lehmann et al. 2000) and reduced N leaching losses compared to under the trees (Lehmann et al. 1999).

A decrease in nodule mass for all three legume cover species in response to increasing NO_3^--N supplied was observed in the glasshouse study. This is consistent with many observations that legume nodulation, as well as legume-rhizobium symbiotic function, is inhibited by NO_3^- (Murray et al. 2016). The results for *C. mucunoides* contrasted with the observation of Camargos and Sodek (2010) that nodule mass and N_2 fixation in this species were unaffected by NO_3^--N, although that was after only a 5 day exposure to NO_3^-, and the same study did report a reduction in nodule number for *C. mucunoides* after 35 days exposure. A simple scoring method to assess nodulation of cover legumes in the field survey indicated poor nodulation for legume cover plants in a few of the older plantations, but generally fair to excellent nodulation across most plantations, irrespective of age and soil nitrate concentration. This was not necessarily expected given the highly acidic nature of the soils (Table 1), and that negative effects of low pH in the root zone on both growth of the legume and activity of rhizobia are well documented (Hungria and Vargas 2000). It is possible that inoculation practices using appropriately adapted rhizobium strains

may have been implemented, but it is more likely that the indigenous rhizobia are effective at nodulation in these soils as has been observed for *C. mucunoides* in low pH soils of the Cerrado in Brazil (Ferreira et al. 2016). Nodule scoring is a useful rapid assessment of the potential N_2 fixing ability of a legume but it is subjective and does not provide any indication of whether nodules are active or moribund. Perhaps this explains why there was no relationship between Ndfa (%) of legume species and nodule score for this field survey. Indeed, it can be concluded from the results of the glasshouse study that nodule mass is probably a better indicator than nodule score or nodule number in relation to pNdfa (%) and the response of legume N_2 fixation to mineral N.

The measures of ammonium in these soils were extremely high and are considered to be an artefact from the mandatory gamma irradiation rather than representative of field conditions. While irradiation is thought to have little effect on NO_3^-concentrations in soils (McNamara et al. 2003), an increase in ammonium might result from amino N released from soil organisms which are all killed during irradiation, with no subsequent conversion to nitrate due to the absence of any active nitrifying bacteria (Lensi et al. 1991). A substantial increase (5 fold) in ammonium concentration in soils has been reported over a 55 day period following similar irradiation (Lensi et al. 1991), particularly where soils were moist. Although the soils for this study had been oven dried at 60°C in PNG it is possible that the samples retained some moisture during that process, or under the humid atmospheric conditions prevalent in PNG the stored samples re-absorbed moisture prior to transport and subsequent treatment in Australia. Delayed release of the imported samples from the Australia irradiation facility meant it was 3 months post-irradiation before the soils were analysed. The effects of ammonium on BNF are not as well defined as those for nitrate (Murray et al. 2016) and may merit consideration in future studies, although clearly valid measures need to be obtained using non-irradiated soils.

The standing shoot biomass (443–792 g/m^2) obtained for legume cover from the field survey for the four plantations 5 years or less in age is similar to that recorded for 3–4 months growth of the same legume cover species in other tropical field environments (Broughton 1977; Sylvester-Bradley 1984; Zaharah et al. 1986; Sanginga et al. 1996a, b; Tian et al. 1999; Giller 2001a; Fosu 2003; Sanginga 2003; Franke et al. 2008; Samedani et al. 2015). Therefore, it could be considered reasonable to assume that amounts of fixed N in standing shoot biomass for each of these four plantations represents 4 months production, and could thus be multiplied by a factor of 3 to obtain annual N_2 fixation estimates of 48–102 kg N /ha/year for legume cover in young PNG plantations. These quantities of fixed N in standing shoot biomass are less than the 150–200 kg N /ha/year reported for the same legume covers under oil palm and rubber plantations in Malaysia (Broughton 1977; Agamuthu and Broughton 1985; Zaharah et al. 1986), but are similar to that for *C. mucunoides* and *M. pruriens* cover crops in Ghana (Fosu 2003). Values reported in the literature appear to be from studies with close to 100% legume cover compared with the lower cover (17–44%) recorded in the present field survey in PNG. A reduced proportion of legume groundcover in plantations is not only due to shading as palm canopy closes, but also the result of other management factors, including poor cover crop establishment, slashing of cover crop vegetation to create harvest paths and removal of plants in the weeded circle. In the PNG survey total standing above-ground biomass (DM) and N content of cover legumes, and by default calculated amounts of N fixed, decreased in plantations more than five years old because legume ground cover markedly decreased in the older plantations. *C caeruleum* was the sole species surviving in plantations > 5 years old, perhaps indicating a greater tolerance to shade and other management influences.

It has been pointed out that the few reported amounts of N fixed by cover legumes in oil palm plantations do not include N in litter (Giller and Fairhurst 2003). Data from the field survey in PNG demonstrate that in plantations less than six years old there can be as much or more DM and N in the litter as there is in the corresponding standing shoot biomass of cover legumes. Indeed, assuming the same pNdfa for litter as shoots, the estimated amount of fixed N in legume litter for young plantations in this study either equalled (5.1-7 kg N/ha, Kapiura estate plantations) or was greater than that in shoots (15.5–19.3 kg N/ha, Mosa estate plantations). Accounting for this fixed N in litter would substantially increase annual estimates of BNF by cover legumes. Furthermore, this litter represents a significant pool of potentially mineralisable N in the warm moist climate of PNG,

and is likely to be a factor in the observed accumulation of soil NO_3^- in the field survey that was related to reduction of pNdfa. Fixed N in legume litter sampled under older plantations in the survey was substantially lower at only 0.1–3.4 kg N/ha, often due to poor groundcover. It should be remembered that these amounts of N are for litter sampled at a single point in time and not annual values which are likely to be greater. Nitrogen transfer to litter from living biomass of cover legumes in plantations (oil palm and rubber) varies with age of cover and has been estimated at 120–160 kg N/ha/year for the first 3 years to less than 40 kg N /ha/year over years 4–7 (Agamuthu and Broughton 1985; Pushparajah and Chew 1998). However, annual litter accumulation and turnover cannot be quantified from the limited measures in this study and clearly requires further research to improve estimates of BNF inputs by cover legumes. Furthermore, a commonality between this study and all others that quantify BNF by tropical perennial cover legumes is that N accumulated below-ground in roots has not been measured. Studies with annual crop legumes have indicated that on average 30% of total plant N may be located below-ground (Herridge et al. 2008), and where legumes are dependent on BNF this represents input of fixed N to the system that needs to be included when considering the effect of cover legumes on the N cycle of oil palm plantations.

Conclusions

This study used a calibrated ureide technique for the first time in oil palm plantations to estimate pNdfa by cover legumes. Proportional dependence on N_2 fixation by legume cover in oil palm plantations in PNG varied widely and was not related to age of the plantation but was strongly negatively correlated with increasing soil nitrate. Total amount of N derived from N_2 fixation in above-ground biomass of legume cover was positively correlated with legume dry matter production which was greatest under young plantations. Legume cover declined with age, due to shading and other management influences, and *C caeruleum* was the only cover species present in plantations that were seven years or older. Further research is recommended, using the calibrated RUN technique to obtain annual estimates of BNF inputs over those obtained simply from standing shoot biomass by quantifying total legume biomass production over time, including litter and root accumulation. With this knowledge, more informed decisions can be made regarding the effective management of N inputs from fertilisers and legumes, to better synchronise fluxes of N in the system, and move towards more sustainable oil palm cultivation.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

The authors have no relevant financial or non-financial interests to disclose.

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