BIOLOGICAL NITROGEN FIXATION BY COVER LEGUMES UNDER OIL PALM PLANTATIONS IN PAPUA NEW GUINEA

A thesis submitted in fulfilment of the requirements for the degree of

Master of Agricultural Science

School of Agriculture, Food and Wine

Faculty of Sciences

The University of Adelaide

Australia

Rachel Pipai

January 2014

Dedicated to the memory of my father Mr Nehemiah Pipai Ndramat

CONTENTS

Abstract

Sustainable management of soil nutrients, and more generally soil health, is a priority concern for the Papua New Guinea oil palm industry, as it is for most other agricultural systems in the world. Like other crops, oil palms need essential elements such as N, P, K and Mg in large amounts annually in order to maintain high fresh fruit bunches (FFB) production. Nutrients are supplied in the form of mineral fertilizers annually to meet nutritional requirements. Legume cover plants used under the oil palm for weed suppression and erosion control also contribute N to the oil palm system through biological N fixation, although amounts of N fixed have not been quantified for these legumes in PNG oil palm plantations. In this study, the xylem ureide technique was calibrated in a glasshouse experiment using ^{15}N isotope dilution for the legume cover species *Calopogonium mucunoides*, *Pueraria phaseoloides* and *Mucuna pruriens*, before being applied in PNG oil palm plantations to assess N_2 fixation by these cover legume species and *Calopogonium caeruleum*. Legume standing shoot biomass under 2 to 25 year old plantations was 144 to 443 g/m^2 and litter was 100 to 804 g/m^2, equating to an estimated mean 400 kg/ha shoot biomass per plantation. Legume shoot N was 3.5 to 12g/m^2 while the litter N was 1.8 to 22 g/m^2 with a mean plantation shoot N estimate of 10 kg/ha. Dependence on N_2 fixation was highly variable, ranging from 18 (*P. phaseoloides*) to 75% (*C. mucunoides*), and did not show any relationship with age of plantation but was significantly lower where soil nitrate-N was high. Amounts of N fixed were 1.5 to 4.4 g/m^2 for standing shoot and 0.9 to 6.0 g/m^2 for litter equating to plantation estimates from 0.3 (*C. mucunoides*) to 34 (*P. phaseoloides)* kg N fixed/ha. These were conservative estimates since the study did not account for N in roots and furthermore only measured standing biomass rather than annual production. Estimates were based on measures of actual percent legume cover (0.6 to 44%) - hence indicated potential for increasing inputs of fixed N by managing for greater cover. Further research is recommended to quantify legume biomass production over time, including litter and root accumulation and turnover. Nevertheless, except for *M. pruriens* which did not transport a large proportion of fixed N as ureides, this study successfully calibrated the ureide technique to quantify input of biologically fixed N from cover legumes in the PNG oil palm system. With this knowledge, more informed decisions can be made regarding the effective management of N inputs from fertilisers and legumes in order to achieve sustainable oil palm cultivation.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Rachel Pipai

Acknowledgments

List of Tables

List of Figures

List of Plates

List of Appendices

1   Introduction

Oil palm (*Elaeis guineensis*) is a tropical perennial tree crop grown in about 43 countries and is now the second most important source of vegetable oil in the world after soybean. It is the only commercially cultivated oil palm species which originated from West and Central Africa, the other species, *Elaeis oleifera*, is originally from Central and South America (Henderson and Osborne 2000). Malaysia and Indonesia alone account for about 80 percent of the world palm oil production. Oil palm was first introduced commercially into Papua New Guinea in 1967 under the nucleus estate-smallholder system (Caudwell 2000; Nelson *et al.* 2010). Under this system a nucleus estate runs large central plantations with surrounding smallholder schemes. These estates are run as joint ventures between the government and a private company. The estate operates the larger plantations, processing mill, and marketing, provides agronomic support to and buys crops from the smallholders. Alienated land in the provinces in which oil palm was being grown such as West New Britain and Oro were opened up for voluntary settlement and eventual oil palm cultivation as part of the industry (Koczberski *et al.* 2001). These smallholdings became known as the 'land settlement scheme' in which each owner had up to 6 hectares of land, 4 of which were expected to be used for oil palm cultivation with the other 2 hectares allocated for housing and gardening purposes. Another form of small scale oil palm cultivation is the 'village oil palm' in which customary land owners are encouraged to cultivate oil palm on their own land, usually 2-4 hectares areas. Now, forty years later oil palm has expanded into new areas covering a total of 130 000 hectares in Papua New Guinea, 55% of which are company owned large-scale plantations and 45% are occupied by smallholders. Totally, this represents only 1% of the world oil palm production but plays a very important role in the PNG economy as the major cash crop earner for the country. In 2008 alone the total export of oil palm was valued at K1 billion (US$344.3 million) comparable to the then national government budget of K6.2 billion (US$2.1 billion). At the same time, over 16,000 people are employed by the oil palm industry while supporting over 18,000 smallholder growers, including an estimated 200,000 people that benefit from oil palm supported households, thus oil palm is a significant economic contributor to PNG (Nelson *et al.* 2010).

There is increasing pressure from consumers and governments for agriculture and food production systems to be sustainable. Increasing oil palm production in PNG places a high demand on the soil resource, especially for supply of nutrients, and there is a need to replenish this in order to have a sustainable oil palm industry. Banabas (2007) reported that oil palm takes out 160-200 kg of N from the soil per hectare per year, which requires input of the same amounts annually in order to maintain sustainable production. Nitrogen fertilizers comprise 60-70% of the total field input costs to meet the nutritional requirements of the oil palm on the N deficient soils in PNG, yet when balancing out how much N was being taken out in the total oil palm stands and harvested crops, 40-60% of the applied fertilizers could not be accounted for. Banabas (2007) was the first to attempt to quantify the loss of N fertiliser under oil palm, and carried out an in-depth study on the N loss pathways in two oil palm growing areas in PNG: Dami on West New Britain and Sangara in Oro province. Soils in these areas have very high infiltration rates being coarse-textured and free draining (West New Britain soils being sandier and coarser in nature while Oro having sandy clay soils), this, combined with the typical high rainfall climate means there is high potential for loss of nutrients through leaching. Banabas (2007) showed that nitrate leaching was the main N loss pathway with further losses via denitrification. Leached nitrate in the tropical environment is likely to move into river systems affecting drinking water quality and causing eutrophication, whilst denitrification will contribute to greenhouse gases.

Legume cover plants such as *Pueraria phaseoloides* and *Calopogonium caeruleum* have long been used, most commonly under the industry managed PNG oil palm plantations, for soil erosion control and weed control and more recently have been recognised for importance as N fixers and a potential source of N for the oil palm in the system (Fairhurst and Hardter 2003). Recently *Calopogonium mucunoides* and *Mucuna pruriens* have also been included in some PNG oil palm plantations. All these cover crops are usually established between palms which are planted in single straight rows during the oil palm planting stage. No attempt has yet been made to assess the amount of N that legume covers are fixing or contributing to the oil palm system in PNG. There is some relevant information provided by a study on sandy clay loam soils in Malaysia which estimated the amount of N fixed by the legumes *Centrosema pubescens* and *P. phaseoloides* to be 150 kg ha^{-1} yr^{-1} under 2-3 year old oil palm (Agamuthu and Broughton 1985). The study used the N balance method to assess N$_2$ fixation as the mean difference in N content between the

vegetation of legume and "natural" covers (Agamuthu and Broughton 1985). Like all methods for assessing N_2 fixation, the N balance method has its disadvantages in that all inputs and outputs of N to the system need to be known or estimated (Unkovich *et al.* 2008) and sometimes lack of all this information reduces the reliability of the method. Nevertheless, if similar amounts of N are being fixed by legume covers under the oil palm in PNG then there is definitely potential via decomposition of the residues for BNF to have input over the longer term into the N balance of the oil palm system.

The Papua New Guinea oil palm industry has committed to become more environmentally sustainable as part of the Roundtable on Sustainable Palm Oil (RSPO). This association is made up of different palm oil stakeholders (producers, buyers, processing companies, retailers, environmental groups, social groups and bankers) with the main purpose 'to develop and implement global standards for sustainable palm oil' (RSPO 2010). One of the main reasons for this underperformance in palm oil production is the reluctance of small holders to purchase fertilizer, especially N-based, due to the high cost. Other factors influencing smallholder production include lack of legume cover and poor block maintenance, especially lack of rigour in weeding, careful placement of frond piles and keeping clearly defined harvest paths. Potentially, an increase in legume cover could have a positive impact on smallholder livelihoods by providing a source of N in the system that will be released as the residues decompose, is cheaper than mineral fertilizers, and could still contribute to improving oil palm production.

To evaluate the environmental balance sheet of legume covers in oil palm systems it is necessary to quantify the inputs of N by biological N_2 fixation and the turnover of legume N to soil organic matter which may ultimately contribute to oil palm N nutrition. This study takes the first step by quantifying N fixation inputs in legume cover crops under oil palm systems in PNG.

2 Literature Review

2.1 Introduction

This literature review provides an overview of the N cycle in ecosystems and details the key processes relevant to cover crops under oil palm plantations – in particular input via BNF as well as the advantages and disadvantages of methods for measuring BNF. The role that legume residue decomposition and associated other soil N processes play in the ultimate provision of an N benefit to the system is also briefly considered.

2.2 Nitrogen Cycle

Nitrogen (N) is an important element in living systems and exists as the most abundant element in the atmosphere (78%) in its gaseous form, dinitrogen (N_2). This N form is unavailable to plants since they use the reduced forms of N (i.e., nitrates, ammonia, ammonium, nitrous oxide etc) for nutrition. The N cycle is a biogeochemical cycle that involves changing of the element nitrogen (N) into its different chemical forms. The main forms of N include the gaseous dinitrogen (N_2), organic N in plants, animals and microorganisms, nitrates, ammonia, ammonium, nitrous oxide and nitric oxides (Erisman *et al.* 2007). These various forms of N are crucial to living organisms. Nitrogen is contained in amino acids, proteins, DNA and RNA (nucleic acids), and it is one of the most essential elements in life (Erisman *et al.* 2007). In plants N is found in the chlorophyll molecule which is needed for photosynthesis and in RuBisCo (Ribulose-1, 5-bisphosphate carboxylase oxygenase), the critical enzyme for photosynthesis. A loss of N in plants results in a loss of chlorophyll (Blackmer *et al.* 1996; Ciompi *et al.* 1996) which in turn affects photosynthesis and eventual food production which is very important for agricultural crops. Important processes in the N cycle include N_2 fixation, ammonification or mineralization, nitrification and denitrification. Certain bacteria aid the conversion of N in the different processes into its different forms and maintain the cycle.

The N cycle in an oil palm system could be represented as shown in Figure 2.1. Nitrogen fertilizers, legume cover, oil palm litter and cattle wastes are recognised sources of N in the PNG oil palm system. Nitrogen fertilizers in the form of ammonium nitrate, ammonium chloride, diammonium phosphate and urea are applied to palms at a rate of 520 to 580 g

N/palm/year to meet the N requirements of the palm (Banabas 2007). A mean of 173 kg N/ha is required annually for oil palm nutrition; 71 kg of which are contained in cut fronds and male inflorescence and recycled in the organic matter. A balance of 102 kg N/ha/year is thus required from other sources. Plantations in PNG apply a mean of 80 kg N/ha/year while 22 kg N/ha/year needs to be sourced from the soil N resources or legume cover.
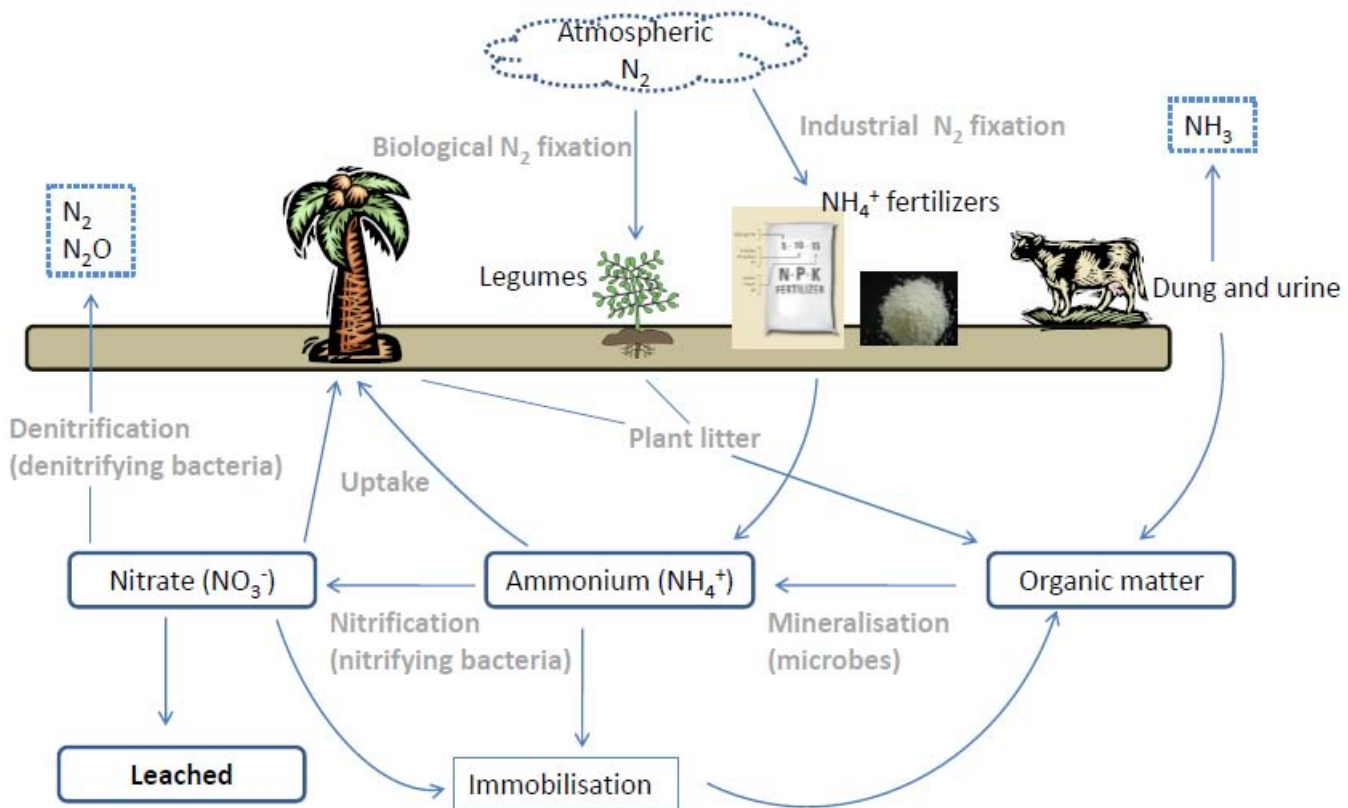
Figure 2.1: The nitrogen cycle

Legume cover plants which are usually established during oil palm planting also contribute to the soil organic matter. As much as 150 kg N/ha/year could be fixed by a mixture of legume cover plants under young oil palm according to Agamuthu and Broughton (1985); this amount is still to be measured for legumes in PNG. Cattle is reared for meat in oil palm plantations referred to as 'half stands' where several rows of palms are left unplanted to allow for grazing. Cattle manure is very high in ammonium and organic N; the latter releases plant available N upon mineralisation (Sorensen *et al.* 2003). Smaller amounts of ammonia present in the cattle manure could be lost through volatilisation while the ammonium could also chemically convert into ammonia as a function of time and temperature (Pennington *et al.* 2009), although none has been measured in PNG oil palm

system. In his study on the N loss pathways in the oil palm system in PNG, Banabas (2007) addressed a lot of the parameters of the N cycle. The rate of nitrification on the fertilizer ammonium chloride (AMC) in Dami and Sangara oil palm plantations were found to be higher in the areas where high organic matter is found; in the frond pile where cut oil palm fronds are usually stacked (Banabas 2007). The half-life ($t_{1/2}$) of ammonium-N before being transformed to nitrate-N was 8-15 days in the frond piles while it was 70 to 80 days in the weeded 1 meter band around the palm base (weeded circle). Banabas (2007) also measured the rate of denitrification from fertilizers to be <1% of the amount of fertilizers applied annually (80 to 150 kg N/ha/year) in the oil palm plantations. The emissions of nitrous gases although insignificant in this amount, could be detrimental in the long run as a greenhouse gas.

2.2.1 Biological nitrogen fixation (BNF)

Biological N_2 fixation (BNF) is the process by which micro-organisms called diazotrophs 'fix' atmospheric nitrogen (N_2) into ammonia (NH_3) (Dilworth and Glenn 1991). Biological N_2 fixation occurs both in the terrestrial and aquatic environments (Zehr and Montoya 2007) and enables the assimilation of N. Nitrogen is contained in amino acids, proteins and chlorophyll molecules, which are the basic building blocks of life, thus the availability of it is vital to life. Biological N_2 fixation is the second most important process in life next to photosynthesis (Unkovich *et al.* 2008). Diazotroph is a term used to describe prokaryotic organisms that can synthesize the enzyme nitrogenase which catalyses the conversion of N_2 to NH_3 (Frank 1982). Diazotrophs require mild temperature and normal atmospheric pressures in order to convert N_2 to NH_3 compared to the industrial (Haber-Bosch) process of making ammonium fertilizers that requires high temperatures and pressures (Frank 1982). These specialized microorganisms include bacteria and blue-green algae which could be free-living (no direct association with plants), symbiotic (as with rhizobia in legume root nodules) or may form associative relationships with higher plants (Dilworth and Glenn 1991). The relationship that is most commonly known and important to agriculture is the symbiotic root nodule-forming relationship between certain legumes and N_2-fixing bacteria from the Rhizobia genera.

Food, fuel, water, clothing and shelter are essential to human survival and it is through agriculture that many, if not all of these needs are met. Hence, there is continual pressure

on agricultural soils to produce these necessities sufficiently. The total world primary food production recorded by FAOSTAT (2013) was 10,678 million metric tonnes in 2010. Cereals such as rice, corn and wheat constitute 23% of the total world primary food supply and are produced in very large quantities annually to meet the rising human demands. Live cattle and sheep world production had a total head count of 2,505 million in 2010 (FAOSTATS 2013). These large productions require large areas of land, so as plantation tree crops. In most cases the same land is used over and over again imposing pressure on the arable properties of the soil. The need to sustainably maintain soil health and soil fertility is now the prime concern in all agricultural systems in the world. Biological N_2 fixation provides a sustainable means to return N into the soil to be available for future crops and is widely utilized in different agriculture systems as part of sustainable agriculture. Much work had been carried out to document the importance of legumes in agriculture as a means to fix atmospheric N_2 and contribute N to agricultural soils. There are food legumes that are used interchangeably as food and also as an N_2 fixer and have played very significant roles in agriculture. More than 60% of grain-legume production is soybeans (Werner and Newton 2005). Certain legumes are used in crop rotation between crop cycles so that the decaying biomass returns captured N into the soil to be available for the subsequent crop; others are used as forage, rotated or planted with pasture grass species; as mulch, or as cover plants to control weed, erosion, pests and fix N_2. These different roles that legumes play will be discussed briefly below.

Amounts of N_2 fixed have also been assessed for a range of shrub legumes (Peoples *et al.* 1996), tree legumes, soybean (Boddey *et al.* 1990; Rennie *et al.* 1988), tropical forage legumes (Gil *et al.* 1997; Viera-Vargas *et al.* 1995), fababean, pea and chickpea (Carranca *et al.* 1999), pasture legumes (Alves *et al.* 2000b; Bolger *et al.* 1995) and many others. Extensive reviews have also been published reporting the amounts of N_2 fixed by commonly grown food legumes, herbaceous forage legumes, tree and shrub legumes, intercropping food legumes with cereals and symbiotic and non-symbiotic associations (Peoples *et al.* 2009a; Peoples and Craswell 1992; Unkovich and Pate 2000).

A large amount of research has been carried out to understand and quantify biological N_2 fixation in both natural (Vitousek *et al.* 2002) and agricultural systems. According to Vitousek et al (2002), the free-living, vascular symbiotic and heterotrophic bacteria fix about $100 kgNha^{-1}yr^{-1}$ in the natural ecosystems. Herridge, Peoples & Boddey (2008)

summarized some estimates of N_2 fixed annually in several agricultural settings by different bacteria groups as shown in Table 2.1.

Table 2.1: A summary of estimates of the amount of N_2 fixed annually by different groups of N fixing bacteria either symbiotic with legumes, associative with non legumes or free-living in agricultural systems (after Herridge, Peoples and Boddey 2008).

Agent	Agricultural system	Area (Mha)	Rate of N_2 fixation (kg N/ha/year)
Legume-rhizobia	Crop (pulse and oilseed) legumes	186	115
	Pasture and fodder legumes	110	110 – 227
Azolla – cyanobacteria,	Rice	150	33
Endophytic, associative & free-living bacteria	Sugar cane	20	25
	Crop lands other than used for legumes and rice	800	<5
	Extensive, tropical savannas primarily used for grazing	1,390	<10

2.2.1.1   Factors affecting BNF

There are many factors that affect biological N_2 fixation including shade, effective rhizobial population, soil nitrate concentration and soil pH and temperature. Shade reduces sunlight and the legumes' ability to manufacture enough carbohydrates to cater for the plant's physiological functions such as growth, metabolism, respiration and N_2 fixation. The rhizobia infecting the roots of legumes need the energy that the host legume makes from photosynthesis in order to fix N_2 in the nodules; a lack of which reduces the activity (Dilworth and Glenn 1984).

Rhizobia exist naturally in most soils in the world but could be totally absent in some, largely due to the soil characteristics such as soil pH, soil temperature and non-N limiting nutrients. Soil acidity, high temperature and moisture deficiency are characteristics of soils in tropical countries and they could affect the plant, roots and rhizobia and affect N_2

fixation (Hungria and Vargas 2000). In southern Europe limited soil moisture and low molybdenum (Mo) in the soil reduces N_2 fixation (Carranca *et al.* 1999), while phosphorus deficient soils reduces legume growth (Fairhurst and Hardter 2003) and N_2 fixation by soybeans (Rennie *et al.* 1988), indicating the need to balance soil nutrients in order to achieve effective N_2 fixation. Different soil rhizobia have different tolerance levels for soil acidity and relatively few could exist in soils with pH below 5.0 (Hungria and Vargas 2000). Alkaline soils with high pH, prolonged flooding and very hot dry soil conditions also affect rhizobial survival (Peoples and Herridge 1990). In areas where soil rhizobia are limited, inoculation is usually carried out to trigger nodulation and eventual N_2 fixation. High root temperatures affect bacterial infection of certain legume species including soybean, peanut, cowpea and beans (Zahran 1999). Some legume species are 'promiscuous ineffective', meaning they can only nodulate with certain rhizobial species and not with just any rhizobia, for example, *P. phaseoloides* (Sylvester-Bradley *et al.* 1991). Inoculating the legumes or the soil in which they are sown with compatible rhizobia is then necessary for symbiotic N_2 fixation to occur.

High soil nitrate also reduces the dependency of legumes on N_2 fixation. Pate et al (1980) showed that higher levels of nitrate applied to nodulating cowpea decreased its dependence on N_2 fixation, as evidenced by the reduction in stem ureides. Voisin et al (2002) illustrated that at higher concentrations of applied mineral N (over 380 kg N ha^{-1}) there was absolute inhibition of symbiotic N_2 fixation in field grown field pea and symbiotic N fixation could not be initiated unless soil nitrate availability dropped below 56 kg N ha^{-1}. Other work has been done as well to show that higher levels of applied mineral N inhibited biological N_2 fixation either in the general inhibition of nodulation (Daimon and Yoshioka 2001; Escuredo *et al.* 1996; Omena-Garcia *et al.* 2011; Pate and Dart 1961; Streeter 1982; Streeter 1985) or in the measure of stem ureides and percentage of atmospheric N in legumes. Legume plants tend to take up soil N in preference to fixing atmospheric N_2 when soil nitrates are available. This legume response to depend less on biological N_2 fixation in the presence of soil nitrate was recently attributed to decreased oxygen supply inside nodules for bacteroid respiration affecting the nitrogenase activity, which nitrate could have influenced by causing increased resistance of O_2 diffusion in the nodule cortex (Lucinski *et al.* 2002).

2.2.2 Non-biological nitrogen fixation

Apart from biological N_2 fixation, atmospheric nitrogen (N_2) can be converted into the biologically available forms through industrial N fixation; combustion of fossil fuels and other less common processes such as lightning strikes. Dinitrogen could also be industrially fixed, through the Haber-Bosch process (Nieder and Benbi 2008). Usually atmospheric N_2 and hydrogen (normally sourced by gas and petroleum mining), with the aid of an iron catalyst, are combined under great pressure and temperature, to form ammonia (NH_3). Most of the NH_3 fixed in this way is used to produce N fertilizers (Nieder and Benbi 2008). To a lesser extent, lightning strikes (Nieder and Benbi 2008) can fix N_2. In this case, N_2 and O_2 under the light energy source are combined to form nitrous oxides (NO_x) which can be available for plants to take up. Only a very small amount of N_2 is fixed this way.

2.2.3 Nitrogen Assimilation, mineralization, nitrification and denitrification

Mineralisation of N from organic matter is a critical component of the N cycle. In agricultural systems, one of the main ways nutrients are returned to the soil is through plant litter. The release or recycling of nutrients from the litter is a function of climate, litter composition (mainly lignin) and abundance of decomposing organisms. Different studies (Couteaux *et al.* 1995; Melillo *et al.* 1982 ; Thomas and Asakawa 1993; Vernon 1978) described how these factors control the rate of litter decomposition. Litter inputs and decomposition in these prostrate perennial cover crops used under oil palms in PNG may be an important factor that affects BNF.

Plants can also naturally take up or assimilate soluble ammonium and nitrates from the soil through their root systems. In ammonification or mineralization, organic N, which is contained in excreted waste or dead plants and animals in the soil, is converted to available mineral forms (NH_4^+, NO_3^-). Bacteria and fungi mediate these biochemical transformations (Malik *et al.* 1985). Nitrification is the process of converting ammonium (NH_4^+) to nitrate (NO_3^-), and it involves two oxidation processes (Burns and Hardy 1975). In soils, the bacteria *Nitrosomonas* and *Nitrobacter* are responsible for converting ammonium to nitrite (NO_2^-) and nitrite to nitrate (NO_3^-) respectively under aerobic conditions. On the other hand, denitrification is the reduction of nitrates (NO_3^+) to oxides of N (e.g. N_2O) and to

nitrogen gas (N_2), although most of the NO_3^+ goes into nitrous gases which pose environmental problems) and completes the N cycle. The bacteria *Pseodomonas* and *Clostridium* carry out this process in anaerobic conditions (Burns and Hardy 1975).

Vitousek et al (1997), Erisman et al (2007), Gruber and Galloway (2008) and Galloway (1998) discussed human activities that have impacted the N cycle, especially in increasing N_2 fixation. High industrial fixation of N_2 (making fertilizers), fossil fuel combustion, the use of N_2-fixing crops and mobilization of N from storage pools (Vitousek *et al.* 2002) are some human activities that accelerate inputs to the terrestrial N cycle.

2.3 Techniques used to assess nitrogen fixation

There are different ways to assess N_2 fixation by legumes and the most appropriate technique depends on the availability of resources. All the techniques available for assessing N_2 fixation have both limitations and advantages as summarized by Unkovich et al (2008). Many reviews have been published on the techniques for measuring biological N_2 fixation (Boddey and Knowles 1987; Peoples and Herridge 1990; Unkovich *et al.* 2008; Unkovich and Pate 2000). Methods include N balance, N difference, acetylene reduction, hydrogen evolution, ureides and ^{15}N- isotope techniques. Nitrogen difference, ureide analysis, ^{15}N natural abundance and ^{15}N enrichment are believed to be the most useful and reliable techniques for quantifying N_2 fixed by legume plants in the field (Peoples *et al.* 2009a).

2.3.1 Xylem ureide N

The xylem ureide method is only applicable for legumes that transport fixed N products in the xylem sap as ureides. Many tropical legume plants from the tribes Phaseoleae and Desmoideae within the subfamily Papilionoideae transport N_2 fixation products in the xylem sap as ureides (Peoples *et al.* 2009b). The principle underlying the xylem ureide technique is that when ureide transporting legumes fix atmospheric N_2 in their nodules, the fixed N is transported in the xylem sap as the ureides allantoin and allantoic acid, while the non-ureide producing legumes transport their fixed N as amides and nitrates alone (Figure 2.2). By measuring the concentration of ureides in the xylem sap relative to the amino-N

and nitrate-N concentrations (which come from soil nitrate uptake) and expressing that as a percentage, commonly referred to as relative-ureide N (RU-N), one could estimate the percentage of N that is derived from the atmosphere (%Ndfa). Since there are four N atoms in the ureide molecule, the relative ureide-N (RU-N) is usually represented as given in *Equation 1*:

Equation 1: $(4a/(4a + b + c)) \times 100$

where *a* is the concentration of ureides in the xylem sap; *b* is the concentration of nitrate-N and *c* is the concentration of amino-N (Herridge 1984).
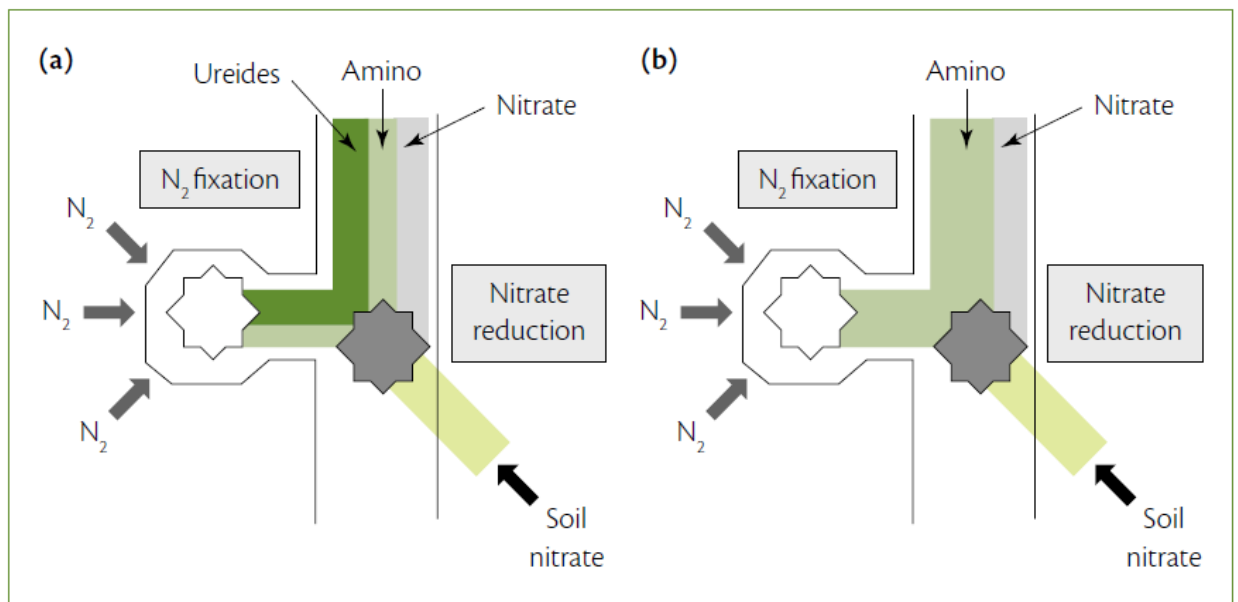
Figure 2.2: When assimilating N, ureide-producing legumes (a) transport three main types of N in the xylem sap with the fixed N transported as ureides while non-ureide producers (b) have only two main forms of N and export fixed N in the form of amides (from Unkovich et al, 2008).

The main advantages of using the ureide technique are that the method is inexpensive and quite simple, so on-farm sampling could be carried out anytime, and many samplings could be carried out at over a relatively short period of time. However, the ureide technique is an indirect measure of %Ndfa and needs to be calibrated with another technique such as the ^{15}N isotope dilution technique (Unkovich *et al.* 2008). Ureide calibrations have been carried out for many legume species as summarised by Unkovich et al (2008); the relationships

between the stem RU-N in these legume species with the %Ndfa derived from ^{15}N isotope dilution were highly correlated proving the reliability of the calibrated ureide technique. A few of the species related to those used under oil palm as cover plants, including *Desmodium ovalifolium* and *Centrosema sp.,* have had ureide calibrations made for them (Table 2.2). Wilson et al (1995) reported that calibrations have also been done for *C. caeruleum*, which is a common legume cover plant used in the PNG oil palm system. Gault and Peoples (1993) reported on *C. caeruleum* that the fully symbiotic (100% N$_2$-fixing) legume showed an RU-N of around 80% while the non-nodulated nitrate-fed plants (0% N$_2$-fixing) had an RU-N of around 10%, again supporting the principles underlying the ureide technique.

Table 2.2: Tropical legume cover plants used under plantation tree crops on which ureide calibrations have been made using ^{15}N isotope dilution (after Unkovich et al 2008).

Species	Calibration equation (y = RU-N; x = %Ndfa)	Sap/tissue[a]
Desmodium ovalifolium	y = 0.89x – 2.1 (sand culture)	Stem
Centrosema sp.	y = 1.04x + 7.43 (soil culture)	Stem
	y = 0.85x – 2.6 (sand culture)	Stem
Desmodium rensonii	y = 0.45x + 18.3	VES

[a]VES, vacuum extracted sap; Stem, extract of dried stem

2.3.2 ^{15}N isotope techniques

Usually ^{15}N isotope techniques are applied to calibrate the ureide technique or they could be used directly to measure N$_2$ fixation. The ^{15}N isotope technique requires the use of very expensive equipment such as a mass spectrometer but the method is believed to be quite reliable (Peoples *et al.* 2009b). Either the natural abundance δ^{15}N (‰) or ^{15}N isotope dilution technique could be applied to calibrate the ureide method. The principles surrounding the ^{15}N isotope techniques involve the isotope of N, ^{15}N. The ^{14}N isotope is the most abundant form of N in the atmosphere while ^{15}N occurs in the atmosphere at a constant abundance of 0.3663 atom%. It was concurred that if the ^{15}N isotopic compositions are different in two sources of N (soil and atmosphere), the proportion of plant N arising from one of them could be determined (Peoples and Herridge 1990). When

the atmospheric N is being taken up, the concentration of ^{15}N would tend towards 0.3663 atom%. The difference in isotopic compositions between the two N sources could be increased by artificially adjusting the soil ^{15}N concentrations (^{15}N enrichment) (Unkovich *et al.* 2008) so the differentiation in the uptake of either could be easily traced.

A reference plant is any plant that is not actively fixing atmospheric N_2 and is normally used to determine the ^{15}N composition of the soil. The reference plant need to exploit that same soil N pool as the legume and have a similar growth duration and pattern of uptake as the legume. In principle, an N_2-fixing legume growing in a medium free of mineral N will have an isotopic composition similar to that of the atmospheric N_2 (Unkovich *et al.* 2008), while a reference plant will have the ^{15}N isotopic composition as the one in the soil medium it is growing in. In situations where soils from the field are used in glasshouse experiments, or the ^{15}N isotope technique is carried out on field soils, the percentage of plant N derived from the atmosphere (%Ndfa) using the ^{15}N isotope technique could then be calculated using *Equation 2*:

Equation 2:

%Ndfa = [1 – (atom% ^{15}N excess N_2-fixing plant)/(atom% ^{15}N excess reference plant)] x 100

For nutrient free media (sand/solution culture) (Boddey and Knowles 1987) and the only N source is enriched ^{15}N, the plant has only two sources of N, the ^{15}N enriched fertilizer and the N derived from the atmosphere via BNF (Boddey and Knowles 1987). The proportion of N derived from the atmosphere (%Ndfa) can then be calculated as shown in *Equation 3*:

Equation 3:

%Ndfa = [1 – (atom% ^{15}N excess in plant/atom% ^{15}N excess in nutrient solution)] x 100

where atom% ^{15}N excess is the ^{15}N composition of the sample above the ^{15}N composition of the atmospheric N_2 (0.3663 atom%).

2.3.3 *15N natural abundance*

The principles underlining the ^{15}N natural abundance technique are similar to all other ^{15}N isotope techniques. The ^{15}N natural abundance technique however takes into account the natural variations in the isotopic compositions of ^{15}N in the soil N and atmosphere N_2 and is represented by the δ unit (Boddey *et al.* 2000; Peoples *et al.* 2009b). The natural ^{15}N abundance is expressed in terms of parts per thousand (δ^{15}N (%$_o$)). The δ values are usually obtained from mass spectrometry and are represented as follows:

Equation 4: δ^{15}N (%$_o$) = (sample atom% - 0.3663/0.3663) x 1000

Since the abundance of ^{15}N in the atmosphere is 0.3663 atom%, δ^{15}N of the atmospheric N_2 is zero.

The ^{15}N natural abundance technique could also be used to measure %Ndfa. *Equation 5* shows how to calculate %Ndfa on legumes that are utilizing both the atmospheric N_2 and soil N:

Equation 5:

%Ndfa = [(δ^{15}N of soil N - δ^{15}N of N_2 fixing legume)/(δ^{15}N of soil N - δ^{15}N of N_2)] x 100

Since reference plants are usually sampled to represent the soil mineral N, *Equation 5* could be re-written as:

Equation 6:

%Ndfa = [(δ^{15}N of reference plant - δ^{15}N of N_2 fixing legume)/(δ^{15}N of reference plant - δ^{15}N of N_2)] x 100

2.4 Tropical legumes and nitrogen fixation

2.4.1 Legumes used for food, mulch and soil improvement

Food legumes include soybean, dry beans, chickpea, lupins, peanuts (or groundnut), other pulses (or grain legumes) and oil seed legumes. The food legumes play a very important role as N fixers as well as a food source for human and livestock. These food legumes could be incorporated into the agricultural systems as monocrops or in crop rotations with other non-legume crops. Either way, the food legumes contribute to soil enrichment with nutrients and also N to the subsequent crops. Much research has been carried out to investigate the benefits of using these food legumes in agriculture. Yoneyama et al (1990) showed that groundnut fixed 85% of its N when not supplied with fertilizer N while in another experiment the groundnut fixed 42-61% N; the cowpeas 33-77% and the soybeans 24-48%, all depending on the amount of fertilizer N applied (Yoneyama *et al.* 1990). Soybean alone fix 16.4 teragrams (16.4 x 10^{12}) of N annually on a global basis, representing 77% of the total N fixed by crop legumes (Herridge *et al.* 2008). Herridge et al (2008) summarized data on a global basis and reported the rate of N_2 fixation for crop legumes to be 115 kg N/ha/year while those of the pasture and fodder legumes 110-227 kg N/ha/yr (Herridge *et al.* 2008). A number of reviews have been made on the amounts of N fixed (kg N/ha/yr) and the proportion of atmospheric N in the total biomass of different food legumes which could replace considerable amounts of N fertilizers (Carranca *et al.* 1999; Herridge *et al.* 2008; Peoples *et al.* 2009a; Peoples and Craswell 1992; Peoples and Herridge 1990; Peoples *et al.* 2009b; Reiter *et al.* 2002; Sanginga 2003; Unkovich and Pate 2000; Wani *et al.* 1995). A summary of some common food legumes and the amounts of N fixed by each of them is given in Table 2.3.

Some non-food herbaceous legumes are grown as cover to enrich the soils such as *M. pruriens* and *P. phaseoloides*. Much work has been done as well to quantify the N benefits of these legumes to subsequent crops and the soil. When grown as a mulching cover, both *M. pruriens* (Sanginga *et al.* 1996b) and *P. phaseoloides* (Tian *et al.* 1999) increased the yield of companion maize and cassava-maize respectively. *P. phaseoloides* increased maize yield by 22-72% and cassava tuber yield by 41% while maintaining the soil organic carbon status better than natural fallows (Tian *et al.* 1999). Similar high yield responses have been observed in maize subsequently grown after the forage legume *Stylosanthes guianensis* and

dual-purpose soybean in West African savannah environment (Franke *et al.* 2008). In the southern region of Brazil, soybean alternates with maize in the summer and black-oats or wheat in the winter (Zotarelli *et al.* 2012) as a green manure/food crop while lupins used as green manure contributed approximately 300 kg N/ha to soil N positively maintaining the N balances for the system (Zotarelli *et al.* 2012). Many similar practices and positive responses have been reported (Agamuthu *et al.* 1981; Broughton 1976b; Koutika *et al.* 2001; Onwu *et al.* 2009; Pongwichian *et al.* 2010; Pypers *et al.* 2005; Ramos *et al.* 2001; Salako *et al.* 2007; Yanggen and Reardon 2001) as well as measurements of how much N has been biologically fixed by different food legumes (Herridge 1982; Herridge and Peoples 1990; Herridge and Peoples 2002a; Herridge and Peoples 2002b; McClure *et al.* 1980; Pate *et al.* 1980; Peoples *et al.* 1989; Purcell *et al.* 1998; Schweiger *et al.* 2012).

Table 2.3: N fixed by some tropical food legumes in different countries reported as proportion of N derived from the atmosphere (%Ndfa) and amount (kg N/ha)

After Peoples & Craswell 1992

Species	Location	N_2 fixed	
		%Ndfa	kg N/ha
Groundnut	Australia	22 – 65	37 - 206
Groundnut	Brazil	47 – 78	68 – 116
Groundnut	India	86 – 92	109 – 152
Pigeon pea	India	88	68 – 88
Soybean	Brazil	70 – 80	85 – 154
Soybean	Hawaii	97 – 80	117 – 237
Soybean	Indonesia	33	26 – 33
Soybean	Thailand	0 - 87	0 – 450
Common bean	Brazil	16 – 71	3 – 32
Common bean	Kenya	16 – 32	17 – 57
Cowpea	Brazil	32 – 70	9 – 51
Cowpea	Indonesia	12 – 33	12 – 22
Cowpea	Kenya	26 – 35	24 – 39
Green gram	Thailand	89 – 90	64 – 66
Black gram	Thailand	95 – 98	119 – 140

2.4.2   Pasture/forage legumes

Legume plants are also integrated into pastures to improve the soil fertility so that pasture grass species could gain nutrients for growth while some legumes are used for forage as well. Cadisch et al (1994) showed the benefits of using the legume cover *C. mucunoides* on a pasture system with *Brachiaria decumbens* where the legume added symbiotically fixed N_2 and also improved net mineralization of litter and root materials increasing plant available N (Cadisch *et al.* 1994). Subterranean clover (*Trifolium subterrraneum)* is an annual pasture legume that plays an important role in the ley farming system in southern Australia by providing fixed N to the pasture system as well as subsequent crops grown in rotation (Bolger *et al.* 1995). The clover fixes between 65-95% N with a range of 50-125 kg fixed N/ha in the system (Sanford *et al.* 1994; Sanford *et al.* 1995). Many other such works have been carried out to improve pastures for cattle grazing (Bolger *et al.* 1995; Henzell *et al.* 1968) and also to quantify the fixed N contribution of different pasture and forage legumes (Alves *et al.* 2000b; Henzell *et al.* 1968; Herridge *et al.* 1996; Peoples *et al.* 1996).

2.4.3   Tropical legume cover plants under tree crops

Tropical and subtropical legumes have long been used both among peasant farmers in the tropics and also in the warmer subtropical countries (Fujita *et al.* 1992). In addition to legumes for food, forage and manure, legumes are also used as cover crops under tropical tree plantations (Giller and Wilson 1991). Soils in the tropics are typically susceptible to leaching due to heavy rains which pose the problem of nutrient losses, mainly N. The use of legume cover plants in recycling N in the system is thus well adapted. Under tropical tree crops various legume cover plants have been used interchangeably for the purpose of weed suppression, soil erosion control, biological control of insect pests and more recently recognized for their input of N via N_2 fixation (Agamuthu and Broughton 1985; Broughton 1976b; Fairhurst and Hardter 2003). Legume species such as *C. caeruleum*, *P. phaseoloides*, *C. mucunoides*, *M. pruriens bracteata* and *C. pubescens* are most commonly used under tropical tree crops such as rubber and oil palm, and to a lesser extent, coconut (Shelton and Stur 1990). Establishing legume covers before the tree canopy closes is important in order to maximize N_2 fixation before legume covers are shaded out which reduces growth rates and N_2 fixation. The above mentioned legume covers are commonly

used under tree crops since they are perennial and remain for some time under trees. *C. mucunoides* and *P. phaseoloides* would normally dominate in the first three years of the plantation tree growth, while *C. caeruleum* and *C. pubescens* persist longer (Shelton and Stur 1990).

Little research work has been done on the amount of N fixed by *P. phaseoloides* under oil palm but it has been used in agriculture with other plants such as the pasture grasses *Brachiaraia humidicola* (Gil *et al.* 1997), and *Brachiaria brizantha* (Viera-Vargas *et al.* 1995), with *Eucalyptus* spp (Mendham *et al.* 2004) and rubber (Broughton 1976a). According to Broughton (1976), a combination of the legume covers *C. mucunoides*, *C. pubescens* and *P. phaseoloides* fixed an average of 150kg N/ha/year during the first five years of a rubber planting, and the maximum amount fixed was about 200kgN/ha/year. Broughton (1976) also showed that these legumes improved soil physical properties such as an increase in the aggregation of finer soil particles, average size of soil aggregates, total soil porosity and soil permeability by water, and led to increased growth of the rubber tree. By the sixth year, all the ground cover virtually died out due to shading (Broughton 1976a). *P. phaseoloides* was also used in a two-year legume-maize crop rotation trial in the savannah environment of West Africa (Franke *et al.* 2008). Natural fallow-maize crop rotation was preferred to a *P. phaseoloides*-maize rotation, due to the positive effect that natural fallow had on maize yield, although there was indication of high N fixation by *P. phaseoloides* (Franke *et al.* 2008).

2.4.4 Legume cover plants used in PNG oil palm plantations

The legume types that are used under the oil palm system in PNG include *C. caeruleum*, *P. phaseoloides*, and more recently *C. mucunoides* and *M. pruriens bracteata*. Legume seeds are usually established during oil palm planting by drilling several lines between adjacent palms along a single row of palms (Fairhurst and Hardter 2003). *M. pruriens bracteata* was recently introduced and only planted in selected areas to monitor its growth but has not been widely used as a legume cover plant in the industry. *Calopogonium caeruleum* is shade tolerant and usually persists long after *P. phaseoloides* die out when the oil palm canopy closes between 5-6 years of age (Giller and Wilson 1991). *P. phaseoloides* is more vigorous in growth and could accumulate higher biomass when first established so the seed

ratio during sowing favours the less vigorous *C. caeruleum* in order to maintain a fair cover until *P. phaseoloides* is shaded out (Fairhurst and Hardter 2003). No work has been done to quantify the amount of N being fixed by these legumes in PNG oil palm systems or other PNG agriculture systems. The current knowledge on the benefits of these legumes stems from the work carried out by Agamuthu and Broughton (1985) in a 2 year old oil palm plantation on a sandy clay loam soil type in Malaysia. They used the N balance method and estimated the amount of N fixed to be around 150 kg N ha^{-1} yr^{-1}. However, soil type and management practices do differ in relation to the Papua New Guinean oil palm environment so the current research will be undertaken to take those factors into account and establish a local knowledge base specifically for PNG oil palm management. The xylem sap ureide method is an appropriate method to assess N$_2$ fixation in the field in Papua New Guinea, as it is relatively cheap to use and reliable. Numerous other work has been undertaken to assess N$_2$ fixation by similar legumes in other agricultural systems (Table 2.4). Some preliminary work (Orrell *et al.* 2009) carried out on nodulation on *P. phaseoloides* and *C. caeruleum* in the Milne Bay province of PNG provides some background knowledge on the legume species. In this study it was observed that nodulation was excellent in 1 – 3 year old plantations while poor nodulation was observed in the older plantations which were more than 6 years old (Table 2.5).

Current knowledge of N use efficiency in PNG oil palm systems is represented by industry records on fertilizer types and rates applied in the plantations, soil analyses, leaf analyses and work done by Banabas (2010). Banabas (2010) recently carried out an in-depth study of the N loss pathways in the PNG oil palm system in West New Britain (study location: Dami) and Oro Provinces (location:Sangara) providing valuable information. Fertilizers applied in the oil palm system comprise mainly ammonium based fertilizers; ammonium nitrate, ammonium chloride and di-ammonium phosphate, and urea. According to Banabas (2007), the amount of time taken for half of the applied ammonium-N to be converted to nitrate-N (via nitrification) depends on the fertilizer rate, the amount of rainfall and where the fertilizers were applied in relation to the oil palm trees. Field experiments by Banabas (2007) to measure the rate of nitrification of fertiliser ammonium-N to nitrate-N found that the time taken for half of the fertilizer to be converted to nitrate was 8-15 days in the frond pile (area under the palms with high organic matter where harvested fronds are piled) and 70-80 days in the weeded circle (a 1m band cleared of weeds around the palm base and intended for fertilizer placement). It was also noted that the most leached form of N was the

nitrate-N, although ammonium-N did leach in very small amounts in Dami only. Banabas (2007) used suction cups buried 150 cm deep in the soil as this zone is beyond the active oil palm root zone, and any nitrate or ammonium N collected at this depth was considered 'leached'. A hand pressure pump connected to the top end of the tubes was used to sample soil water for analysis the day after every rainfall over a one year period (2004 to 2005). Banabas (2007) used the suction cups and deep drainage estimates from water balance measurements and estimated that annual N losses via leaching in the two PNG sites he measured (Dami and Sangara) were 96 and 20 kg N/ha/year. These values were very high compared to Chang and Abas (1986) who estimated leaching from tropical oil palm systems to be 12 kg N/ha/year. The evidence of leached N in these studies necessitates the need to sustainably manage nutrients in the oil palm system.

Table 2.4: Amounts of N fixed in tropical legumes commonly used as cover plants under plantation tree crops

Legume type	Technique used	%Ndfa	N fixed kg/ha/year	Reference
C. mucunoides	[15]N natural abundance	65	59	(Fosu 2003)
	[15]N natural abundance	35 – 79	1 - 76	(Giller 2001a)
	N difference		136-182	(Giller 2001b)
C. mucunoides+P. phaseoloides	N balance		136 - 182	(Reynolds 1982)
C. mucunoides mucunoide+P. phaseoloides phaseoloide+ C. pubescens	N balance		150	(Broughton 1976b)
C. pubescens	[15]N dilution	65 - 83	19 & 41	(Cadisch *et al.* 1989)
C. pubescens+P. phaseoloides	N balance		150	(Agamuthu and Broughton 1985)
Desmodium ovalifolium	[15]N dilution	44 - 70	11 & 25	(Cadisch *et al.* 1989)
M. pruriens	[15]N dilution	70	166 - 313	(Sanginga *et al.* 1996a)
	not given		133 - 188	(Sanginga 2003)
	not given		37 - 69	(Sanginga 2003)
	[15]N natural abundance	60 - 83	18 - 213	(Giller 2001a)
	[15]N natural abundance	81	106	(Fosu 2003)
P. phaseoloides	[15]N dilution	68 - 87	49 & 115	(Cadisch *et al.* 1989)
	[15]N dilution	54		(Gil *et al.* 1997)
	[15]N dilution and N difference	34 - 61		(Viera-Vargas *et al.* 1995)
	[15]N dilution	80	38*	(Zaharah *et al.* 1986)
	[15]N natural abundance	44 - 61	6-53	(Giller 2001a)
	[15]N dilution	88	115	(Giller 2001b)

* measured for 3 months only

Table 2.5: Nodule scoring of the average number of active and ineffective nodules on a mixture of P. phaseoloides and C. caeruleum plants (total of 12 plants) under different oil palm ages in Milne Bay Province, Papua New Guinea (after Orrell et al 2009).

Age (years)	0 – 5 cm root depth		>5 cm root depth		Score	Interpretation
	Active nodules	Ineffective nodules	Active nodules	Ineffective nodules		
1 – 3	64	69	40	54	4 – 5	Excellent nodulation & excellent potential for N_2 fixation
4 – 8	0	0	0	12	0	Poor nodulation. Little to no N_2 fixation
9 – 13	0	0	1	10	0	Poor nodulation. Little to no N_2 fixation
19 – 25	0	0	0	16	0	Poor nodulation. Little to no N_2 fixation

2.5 Conclusion

Whilst it is clear from measurements made elsewhere that there is potential for input of N via cover legumes a key knowledge gap for PNG is the lack of any local measurements relevant to the management systems and cover legumes currently being used. Assessment of N_2 fixation by legume cover plants under oil palm in PNG has not been undertaken before but it is possible that they could be responsible for substantial inputs of N to the system. Based on the work done by Agamuthu and Broughton (1985) and Banabas (2010) on N_2 fixation rates and N pathways respectively, and the numerous assessments carried out on the legume cover plants *C. mucunoides, P. phaseoloides, M. pruriens and C. caeruleum,* this research is undertaken to assess N_2 fixation under oil palm in PNG. It is hypothesised that the xylem sap ureide method is appropriate to assess N_2 fixation in the field in Papua New Guinea as it is relatively cheap and easy as an on-farm technique, and the legumes to be investigated are likely to be ureide exporters. A glasshouse experiment is carried out to calibrate the xylem sap ureide technique for these legume cover plants using the ^{15}N isotope dilution technique. The outcome of this research will benefit the oil palm industry in PNG directly, and in particular the smallholder oil palm growers who need to increase fresh fruit bunch (FFB) production to higher and expected levels. By quantifying the amount of N

fixed per hectare per year by legume cover, the use of legume cover could be encouraged and maximized in the smallholders and the oil palm companies. By understanding how much N these legumes are fixing under the oil palm, better informed decisions could be made to integrate legumes as a sustainable N source. This should eventually replace a considerable amount of mineral N fertilizer inputs and in turn support environmental sustainability and also reduce fertilizer costs.

3 Calibration of the xylem ureide technique using ^{15}N isotope dilution for the tropical legumes *Calopogonium mucunoides, Pueraria phaseoloides* and *Mucuna pruriens*

3.1 Introduction

Measurement of N_2 fixation by legume cover plants using the xylem ureide technique requires calibration of the ureide assay for the relevant species against another method that measures N_2 fixation (Unkovich *et al.* 2008). A glasshouse study was thus carried out with the aims to a) use the ^{15}N isotope dilution technique to calibrate the xylem sap ureide technique in three legume cover species: *Calopogonium mucunoides*, *Pueraria phaseoloides* and *Mucuna pruriens*, and b) to observe the effect of different concentrations of nitrate fed to the plants on growth and N relations of legume shoots, roots and nodules.

3.2 Materials and methods

3.2.1 Glasshouse experiment

The glasshouse experiment was conducted from May 2011 to January 2012 at the Waite campus of the University of Adelaide in South Australia (Plate 1). The glasshouse temperature had an average of 25°C during the day throughout summer and winter and had an average minimum of 17°C low during winter nights. Natural lighting conditions were allowed and during winter glasshouse lights were switched on to assist photosynthesis during the daytime.

Seeds were sourced from the Australian Tropical Grains Germplasm Centre in Queensland for the three legume cover species of interest: *C. mucunoides*, *P. phaseoloides* and *M. pruriens*. The *C. mucunoides* and *P. phaseoloides* seeds both have hard seed coat and needed to be scarified before they could germinate. *C. mucunoides* seeds were soaked in hot (75°C) water for three minutes to break the dormancy (Giller and Fairhurst 2003), while *P. phaseoloides* seeds were soaked for one hour in glycerol at 50°C. *M. pruriens* seeds germinated without scarification. Seeds were sown in free-draining 4 litre pots containing very fine acid-washed river sand from Dry Creek, SA, in May 2011 (*C. mucunoides* and *M. pruriens*) and July 2011 (*P. phaseoloides*). All seeds

were inoculated, with a Group M *Bradyrhizobium* strain (CB756) obtained from Beckerwood, Australia, by mixing peat inoculum in water and pouring a small amount of slurry into the seed holes before the seeds were sown.

The glasshouse experiment consisted of four nitrate treatments and a control (zero NO_3), four replicates and two harvests for each species giving a total of 120 pots arranged in a randomized complete block design. The basal nutrient solution was developed to suit plant requirements based on the formulation by Herridge and Peoples (2002) . The basal nutrients (also the control) in grams per litre (g/L) consisted of: 0.0625 $MgSO_4$, 0.0368 $CaCl_2$, 0.02188 KH_2PO_4, 0.0188 KCl, and 0.0259 FeEDTA. Trace elements in milligrams per litre (mg/L) consisted of: 0.715 H_3BO_4, 0.4525 $MnCl_2$, 0.0275 $ZnCl_2$, 0.0125 $CuCl_2$ and 0.0063 $NaMoO_4$. Labelled potassium nitrate (5.0 atom% ^{15}N) was mixed with unlabelled $Ca(NO_3)_2.4H_2O$, KNO_3 to make up 1, 3, 5 and 10 mM NO_3. This resulted in ^{15}N enrichments of: 1 atom% excess (1 mM NO_3), 0.33 atom% excess (3 mM NO_3), 0.2 atom% excess (5 mM NO_3) and 0.1 atom% excess (10 mM NO_3). Pots were watered as needed with tap water until the first true leaves were observed on the plants and then supplemented with basal nutrient solution. The different species grew at different rates and so nitrate treatments were imposed at different times after sowing and at different strengths (Table 3.1). At ten weeks after sowing, *M. pruriens* plants received 1 litre of the full strength treatments twice per week, and *C. mucunoides* and *P. phaseoloides* received at the same rate one-eighth strength of the different treatments, the latter changed to a quarter strength two weeks later. *C. mucunoides* received the full-strength treatments at 20 weeks after sowing and *P. phaseoloides* at 13 weeks after sowing, both at 1 L per pot twice a week until harvest.

Legume species were harvested prior to flowering when they had produced reasonably comparable vegetative growth per plant to that observed in the field, and again five weeks later. *M. pruriens* were harvested at sixteen weeks after sowing in September 2011 and 5 weeks later in October 2011. *C. mucunoides* and *P. phaseoloides* species were first harvested in December 2011, at 29 weeks and 23 weeks after sowing respectively, and five weeks later in January 2012 (Table 3.1).
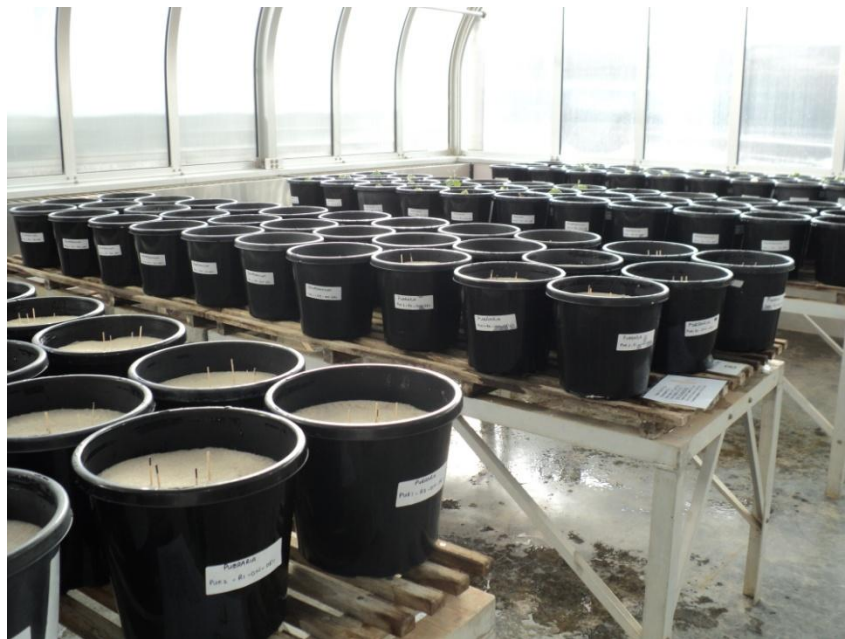
Table 3.1: Time after sowing (in weeks) when N treatments were applied and plants were sampled

	M. pruriens	*Calopogonium*	*P. phaseoloides*
1/8th strength	-	10	10
¼ strength	-	12	-
Full strength	10	13	20
Harvest 1	16	29	23
Harvest 2	21	34	28

During harvest, each plant was cut just above the sand surface and 7 stem pieces from each plant, measuring between 12 and 15cm long, were excised and oven dried at 85°C for 48 hours and weighed, as was the remainder of the shoot. The intact root system was left for up to 1 hour, allowing time for the xylem sap to emerge from the cut stump under root pressure. A 20-200µL pipette was used to collect exuded sap into 1.5mL Eppendorf tubes, which were stored in an ice-filled container before being transferred to a freezer for storage until analysis. After the xylem sap collection, each pot was tipped over carefully to keep the roots and nodules as intact as possible. Roots were washed in clean tap water and collected. A 2mm sieve was used to capture the remaining roots and nodules from the wash bucket. Each root was divided into 0-5cm, and >5 cm sections in relation to sand depth. Nodules were separated from the root and counted before roots and nodules were oven-dried at 85°C for 48 hours and weighed. All the dried plant materials were then ground separately in a ball mill grinder before analysis. Legume shoots, roots and nodules were sent to the University of Western Australia for ^{15}N and total N analysis, while the stem and sap samples were analysed for relative ureide-N as described below.

3.2.2   Laboratory analyses

3.2.2.1   Analysis of xylem sap in stem segments

All ureide analysis was conducted following the protocol outlined in Unkovich et al (2008). The ground stem segments were first subjected to solute extraction. Distilled water (25mL) was added to 0.5 g of ground stem material contained in 25mLs Erlenmeyer flasks, allowed to sit in heated sand to boil for 2 minutes, filtered while hot through 15cm Whatman No. 40 filter paper and collected in 50 mL volumetric flasks. The volume of filtrate was made up to 50 mL with distilled water and stored frozen until analysis.

To calculate the relative ureide-N in the xylem sap of legume plants required the analysis of ureide-N, amino-N and nitrate-N. The ureide-N was analysed using the Rimini-Schryver reaction as described by Young and Conway (1942), where allantoin is hydrolysed by an alkali to allantoic acid, which is further hydrolysed by acid to urea and glyoxylic acid. Ureide was analysed as the phenylhydrazone of glyoxylic acid. The amino compounds were analysed using the ninhydrin method described by Yemm and Cocking (1955). Coupled deamination of the amino acid to ammonia and the reduction of ninhydrin to hydrindantin, and the further condensation of both products form a purple colour, which is attributed to the anion of the diketohydrindylidenediketohydrindamine (DYDA). The nitrate compounds were analysed using the salicylic acid method of Cataldo et al (1975, see also Unkovich et al (2008)).

3.2.2.1.1   *Ureide analysis*

Twenty xylem sap or stem extracts, three water blanks, five standard allantoin concentrations with their duplicates (0, 0.01, 0.02, 0.04 and 0.10 mM) and three internal standards which consisted of the first three allantoin standard concentrations were analysed per batch. Standard allantoin concentrations were made up using a 1.0 mM allantoin solution which was prepared on the same day using 39.53mg of allantoin dissolved in 250 mLs distilled water. Allantoin standards were made up as indicated in Appendix 1.

Each stem or sap sample, 0.5 or 0.1 mL respectively, was made up to 2.5 mL total volume in a test tube using distilled water. 0.5 mL 0.5N NaOH was added to each tube and the test tube rack was placed in boiling water for 10 minutes. Racks were taken out and 1.0 mL of 0.65N HCl/0.33% phenhydrazine was added to each test tube before returning the rack to the boiling water for another 2 minutes. Racks were then plunged immediately into an ice-bath for 15 minutes. Then 2.5 mL of a 10N HCl/1.67% KFeCN mixture was added to the test tubes in such a way that the contents mixed evenly, and tubes were allowed to stand for 10 minutes for the expected red colour development. The optical density (O.D.) was quickly read at 525 nm on a GBC UV/VIS 916 spectrophotometer and GBC FS3000 autosampler. The whole O.D. reading needed to be completed within 15 minutes, after which the red colour disappeared.

3.2.2.1.2   Amino-N analysis

The same number (36) of samples was analysed per batch as in the ureide analysis: 20 stem or sap samples, five standard amino concentrations and their duplicates (0.00, 0.10, 0.20, 0.40 and 1.00 mM), three internal standards (0.00, 0.10 and 0.20 mM) and three water blanks. Standard amino concentrations were made up using a 2.0 mM amino-N stock solution which was prepared on the same day using 66mg asparagin (Asp) and 73mg glutamine (Gln) dissolved in 500 mL distilled water. Different standard concentrations were made up according to Appendix 1.

From each sample, 1.0 mL was measured into a test tube, then 0.5 mL of citrate buffer was added to it, and then 1.2 mLs of ninhydrin reagent was added before placing the test tubes rack in the boiling water for 15 minutes. The rack was returned to the bench after that time, and 3.0 mLs of 60% ethanol was added using a pipette to the test tubes quickly causing the contents to mix. An expected blue color development was observed before the OD was read at 570 nm.

3.2.2.1.3   Nitrate analysis

A total of 38 samples were analysed in a single batch for nitrate determination: 20 sap or stem samples, 6 standard nitrate concentrations for a standard curve (0, 1.25, 2.5, 5.0, 10.0 and 15.0 mM nitrate) and their duplicates, 3 internal standards (0, 1.25 and 2.5 mM nitrate) and 3 water blanks. Nitrate standards were made up using a 25 mM nitrate stock solution which was prepared by adding 632 mg of KNO3 to 250 mls of distilled water. The different standard concentrations were made up according to Appendix 1.

During analysis, 0.05 mLs of each sample was pipetted into test tubes, then 0.20 mL of a mixture of salicylic/sulphuric acid, made by adding 5g of salicylic acid to 100 mLs of concentrated sulphuric acid, was added and the test tubes left to stand for 20 minutes. After that, 4.75 mLs of 2N NaOH was added and the samples were allowed to stand for another 10 minutes to obtain an expected yellow colour before reading the O.D. at 410 nm.

3.2.2.1.4   Standard curves

From each of the ureide, amino and nitrate analysis, standard curves were constructed using the standard sample concentrations along the horizontal (x) axis and the optical density readings as the vertical (y) axis. Each graph equation from the different analyses was used to determine the concentrations of the ureide, amino and nitrate respectively in the sap or stem samples in order to calculate the relative ureide-N (RU-N).

3.2.2.1.5 ^{15}N and total N analysis

The ground shoots, roots and nodules of the legume plants were analysed for ^{15}N and total N at the University of Western Australia using an automated combustion analyser linked to a mass spectrometer - the Europa Scientific ANCA NT System, Solid/Liquid Preparation Module (Dawson and Brooks 2001).

Statistical analysis

The analysis of variance (ANOVA) was used to test the significance of varying nitrate treatments on legume growth and N_2 fixation while the significance of the relationship between stem relative ureide-N and %Nda was assessed using correlation analysis. A strong linear correlation between stem RU-N (%) and %Ndfa provided the calibrated equation necessary to assess nitrogen fixation by the different legume species.

3.3   Results

3.3.1   Calibration of relative ureide-N (RU-N) with proportional dependence on N_2 fixation assessed by the ^{15}N isotope dilution technique

3.3.1.1   Relative ureide-N (RU-N %)

The means of relative ureide-N in the different legume species combined from two harvests with statistical tests are shown in Table 3.2. The results from individual harvests are presented in Appendix 3. There was an obvious decrease in the stem RU-N with increasing nitrate in all three species (Figure 3.1). The decrease was significant in *C. mucunoides* (P<0.001) and *P. phaseoloides* (P<0.001) while there was no significant difference (P=0.09) in stem RU-N in *M. pruriens* with increasing nitrate concentration. The highest record of stem RU-N for *M. pruriens* was 12.1% (zero nitrate) and the lowest was 2.9% (10

mM nitrate) with an overall mean of 9.8%. *C. mucunoides* and *P. phaseoloides*, on the other hand, had higher RU-N in stems (63.6 and 68.3% respectively) at zero nitrate. As the amount of nitrate fed to these legumes increased, the stem RU-N in *C. mucunoides* and *P. phaseoloides* dropped significantly to be similar to *M. pruriens* and thus there was no significant difference between each species at 3 mM, 5 mM and 10 mM nitrate.
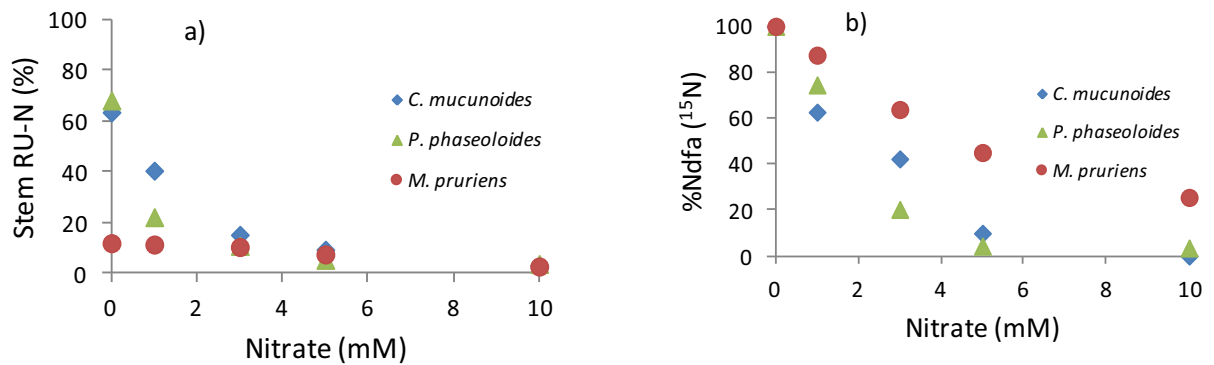
Figure 3.1: Mean of (a) relative ureide-N (%) and (b) %Ndfa (derived from ^{15}N analysis) from two harvests for C. mucunoides, M. pruriens and P. phaseoloides at different nitrate concentrations

Table 3.2: Effect of nitrate treatments on mean stem RU-N (%) and percentage of N derived from the atmosphere (%Ndfa) across two harvests in C. mucunoides, M. pruriens and P. phaseoloides and a two-way ANOVA on the effect of nitrate between each species with significance levels at P≤0.05.

Nitrate (mM)	Stem RU-N (%)				%Ndfa			
	C. mucunoides	*M. pruriens*	*P. phaseoloides*	Species x Treatment	*C. mucunoides*	*M. pruriens*	*P. phaseoloides*	Species x Treatment
0	63.6	12.1	68.3		100.0	100.0	100.0	
1	40.6	11.5	22.3	P<0.001	62.7	87.5	74.5	P<0.001
3	15.4	10.6	11.0	LSD$_{0.05}$ = 14.4	42.3	63.8	20.4	LSD$_{0.05}$ = 12.0
5	9.5	7.6	5.5	CV% = 75.3	10.0	45.1	4.5	CV% = 24.5
10	3.3	2.9	3.9		0.1	25.6	3.6	
	P<0.001	NS	P<0.001		P<0.001	P<0.001	P<0.001	
Grand mean	26.5	8.9	22.2		43.0	64.4	40.6	
LSD$_{0.05}$	21.1		12.2		17.8	8.6	7.9	
CV%	78.4		54.1		40.6	13.2	19	

3.3.1.2   Nitrogen fixation measured by ^{15}N isotope dilution

The percentage of N derived from the atmosphere (%Ndfa) is by default 100% at zero nitrate for all legume species (Table 3.2) and the ^{15}N values ranged from 0.3651- 0.3669. The mean %Ndfa across the two harvests decreased with increasing nitrate in all species from zero to 5 mM nitrate and still decreased significantly at 10 mM nitrate in *M. pruriens* (Figure 3.2). At 5 mM nitrate the %Ndfa in *C. mucunoides* (10%) and *P. phaseoloides* (4.5%) were not significantly different to the %Ndfa at 10 mM nitrate (0.1 and 3.6% respectively). *M. pruriens* maintained a significantly higher (P<0.001) %Ndfa at 5 mM nitrate than at 10 mM nitrate compared to *C. mucunoides* and *P. phaseoloides*.
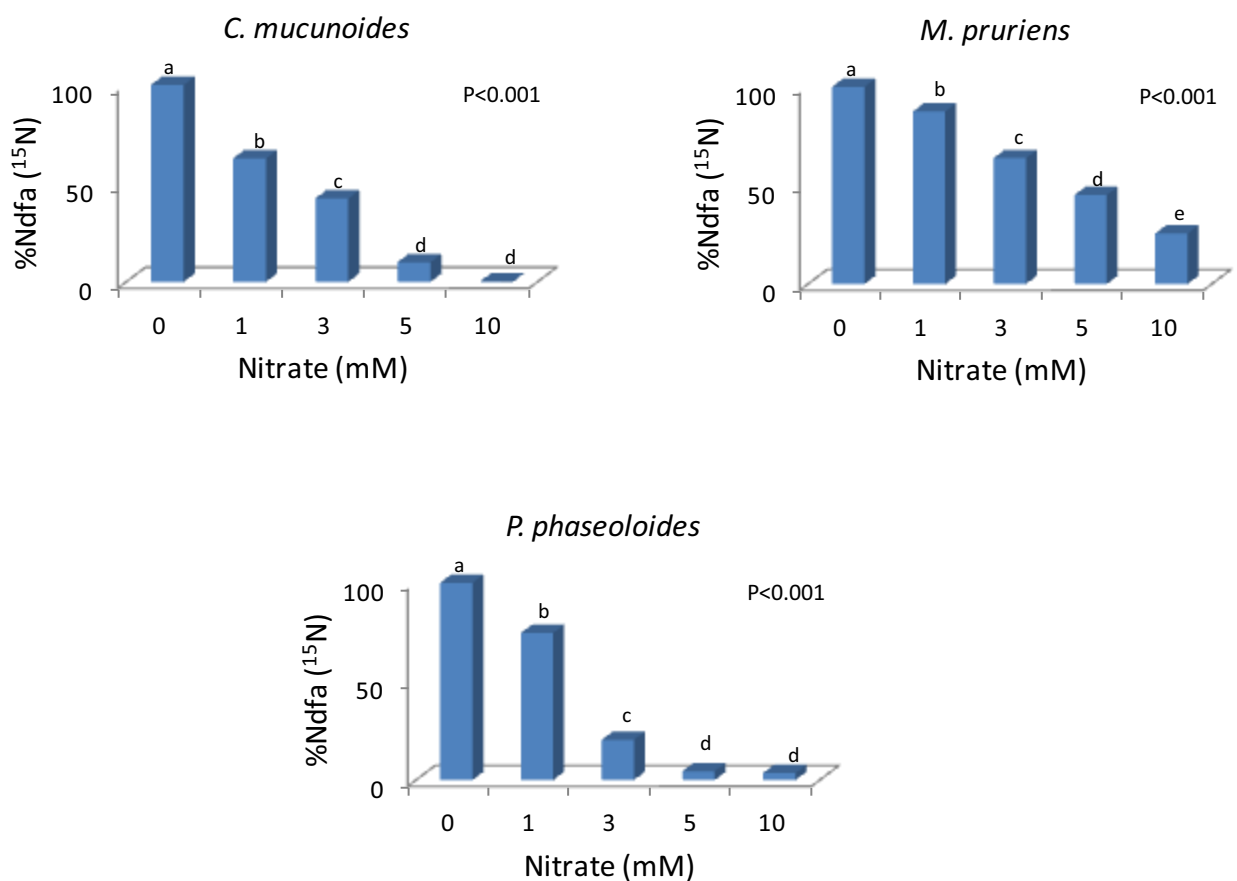
Figure 3.2: Effect of nitrate on the %Ndfa (mean of 2 harvests) in C. mucunoides, M. pruriens and P. phaseoloides. Letters indicate the lsd's at P=0.05 and bars with similar letters are not significantly different from each other.

3.3.1.3   Relationship between stem RU-N and %Ndfa

Regression analyses of stem RU-N (%) and %Ndfa from the ^{15}N method (mean of two harvests) provided calibration curves for *C. mucunoides*, *M. pruriens* and *P. phaseoloides* (Figure 3.3). Stem RU-N and %Ndfa were highly correlated (R^2=0.7 and 0.8) in *C. mucunoides* and *P. phaseoloides* respectively. *M. pruriens*, on the other hand, had stem RU-N and %Ndfa values that were only weakly correlated (R^2=0.4). The regression equations arrived at were: y=0.5971x + 0.7882 for *C. mucunoides*, y=0.5374 + 0.3806 for *P. phaseoloides* and y=0.1311x + 0.5023 for *M. pruriens*.
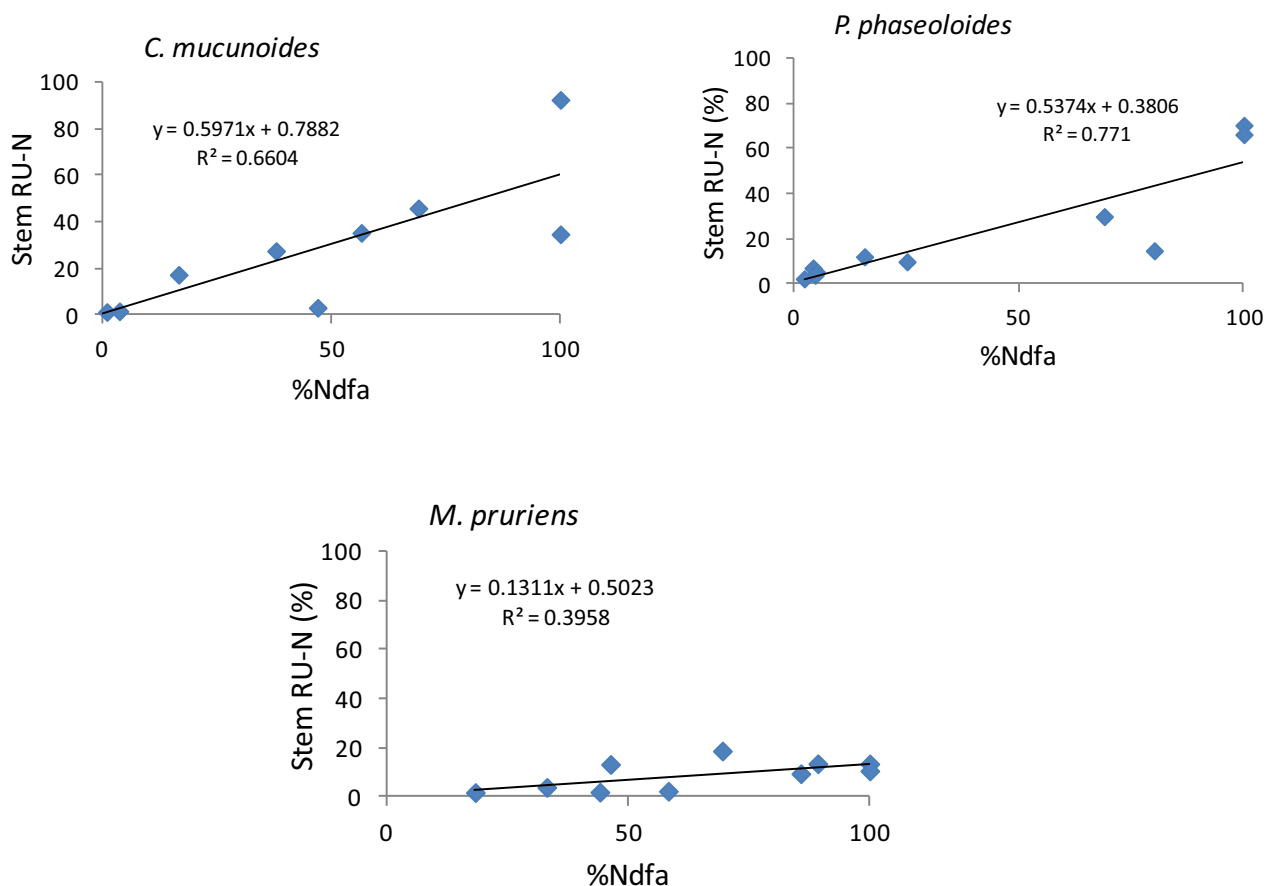
Figure 3.3: Correlation and regression of the RU-N and %Ndfa (^{15}N isotope dilution technique) from mean of two harvests for the legume cover species C. mucunoides, M. pruriens and P. phaseoloides.

3.3.2 Nitrate effects on plant growth and N content of legumes

3.3.2.1 Plant dry weight

The different nitrate treatments did not have a significant effect on the dry weights of nodule, root, shoot or total plant dry weights in *C. mucunoides* in either the first or second harvests (Appendix 6) or on their average across harvests (Appendix 8). The average nodule, root and shoot dry weights in the first harvest were 0.4, 3.5 and 15.3 g/plant respectively. The grand means of nodules, roots, shoot and plant dry weights for *C. mucunoides* at the second harvest (0.4, 3.7, 15.4 and 19.5 g/plant respectively) were also very similar to those in harvest 1 and there was no significant difference between the two harvests.

At the first *M. pruriens* harvest nodule dry weight in 1 mM nitrate (2.2g/plant) was significantly higher than that in 5 mM (1.5g/plant) and 10 mM (1.0g/plant) nitrate (Figure 3.4). There was no significant difference in root dry weight between the nitrate treatments. The shoot dry weight was significantly higher in 10 mM nitrate (32g/plant) than zero (17.1g/plant) and 1 mM (20.5g/plant) nitrate but not significantly higher than 3 and 5 mM nitrate. Shoot dry weight in 3 mM nitrate (28.8g/plant) was also significantly higher than zero nitrate. The *M. pruriens* total plant dry weight in 10 mM nitrate (36.5g/plant) was significantly higher than zero (21.5g/plant) and 1 mM (25.2g/plant) nitrate, and the plant dry weight in 3 (33.4 g/plant) and 5 mM (30.3 g/plant) nitrate were also significantly higher than zero nitrate.

At the second harvest of *M. pruriens*, the nodule dry weight in zero and 1 mM nitrate treatments were similar (4.8g/plant) and were significantly higher than in 3 (2.1), 5 (2.5) and 10 (1.0 g/plant) mM nitrate. *M. pruriens* nodule dry weight in 5 mM nitrate was also significantly higher than 10 mM nitrate (Appendix 6). Its shoot dry weight was the highest in 1 mM nitrate (52.8 g/plant) and was significantly higher than in zero (34.1) and 10 mM (28.4) nitrate. Shoot dry weight in zero nitrate was not significantly different to 10 mM nitrate. *M. pruriens* plant total dry weight was the highest in 1 mM nitrate (67.0 g/plant) and was significantly different to shoot dry weight in 3 (44.3) and 10 (36.2) mM nitrate. The differences in root dry weight between the different nitrate treatments were not significant. The average plant dry weight of harvest 2 (51.6 g/plant) was significantly higher ($P=0.004$) than of harvest 1 (29.4 g/plant) (Appendix 8).

The dry weights of nodules, roots and shoots of *P. phaseoloides* at the different nitrate concentrations with the statistical tests are presented in Appendix 6. In the first harvest nodule dry weight of *P. phaseoloides* in zero nitrate (1.3 g/plant) was significantly higher than in the other nitrate treatments (Figure 3.4). Root dry weight in *P. phaseoloides* at 10 mM nitrate (4.7 g/plant) was not significantly higher than at zero nitrate (3.1 g/plant), but it was significantly higher than at 1 (1.6 g/plant), 3 (1.7 g/plant) and 5 mM (0.9) nitrate. The shoot dry weight was higher in 10 mM nitrate than the other treatments. Total plant dry weight at 10 mM nitrate (31 g/plant) and zero mM nitrate (18.1 g/plant) were not significantly different from each other but the former was significantly higher than 1, 3 and 5 mM nitrate. Nodule dry weight for *P. phaseoloides* in the second harvest was significantly higher in 0 and 1 mM nitrate treatment (1.6 and 2.1 g/plant respectively) than in the higher nitrate treatments.

P. phaseoloides shoot dry weight was highest in 1 mM nitrate (24.9 g/plant) and was significantly higher than 5 (7.0) and 10 (10.6), but was not significantly different to zero (17.9) and 3 (16.4) mM nitrate. The highest plant total dry weight was 32.8 g/plant (1 mM nitrate) which was significantly higher than plant dry weight in 5 (8.7) and 10 (12.3) mM nitrate. *P. phaseoloides* plant dry weight in zero, 1 and 3 mM nitrate were not significantly different from each other. The root dry weights between the nitrate treatments were not significantly different from each other. The plant dry weight in 1 mM nitrate in Harvest 2 (32.8 g/plant), however, was significantly higher than in harvest 1 (9.2 g/plant), while in 10 mM nitrate, the plant dry weight in Harvest 2 (12.3) was significantly lower than harvest 1 (31.0 g/plant). Between harvests 1 and 2 the grand mean of plant dry weights (14.7 and 19.7 respectively) for *P. phaseoloides* were not significantly different from each other.

When comparing the mean plant dry weights of the three legume species, *M. pruriens* had significantly higher plant dry weights (29.4 and 51.6g/plant) than *C. mucunoides* (19.2 and 19.5) and *P. phaseoloides* (14.7 and 19.7) in both harvests (Appendix 6).
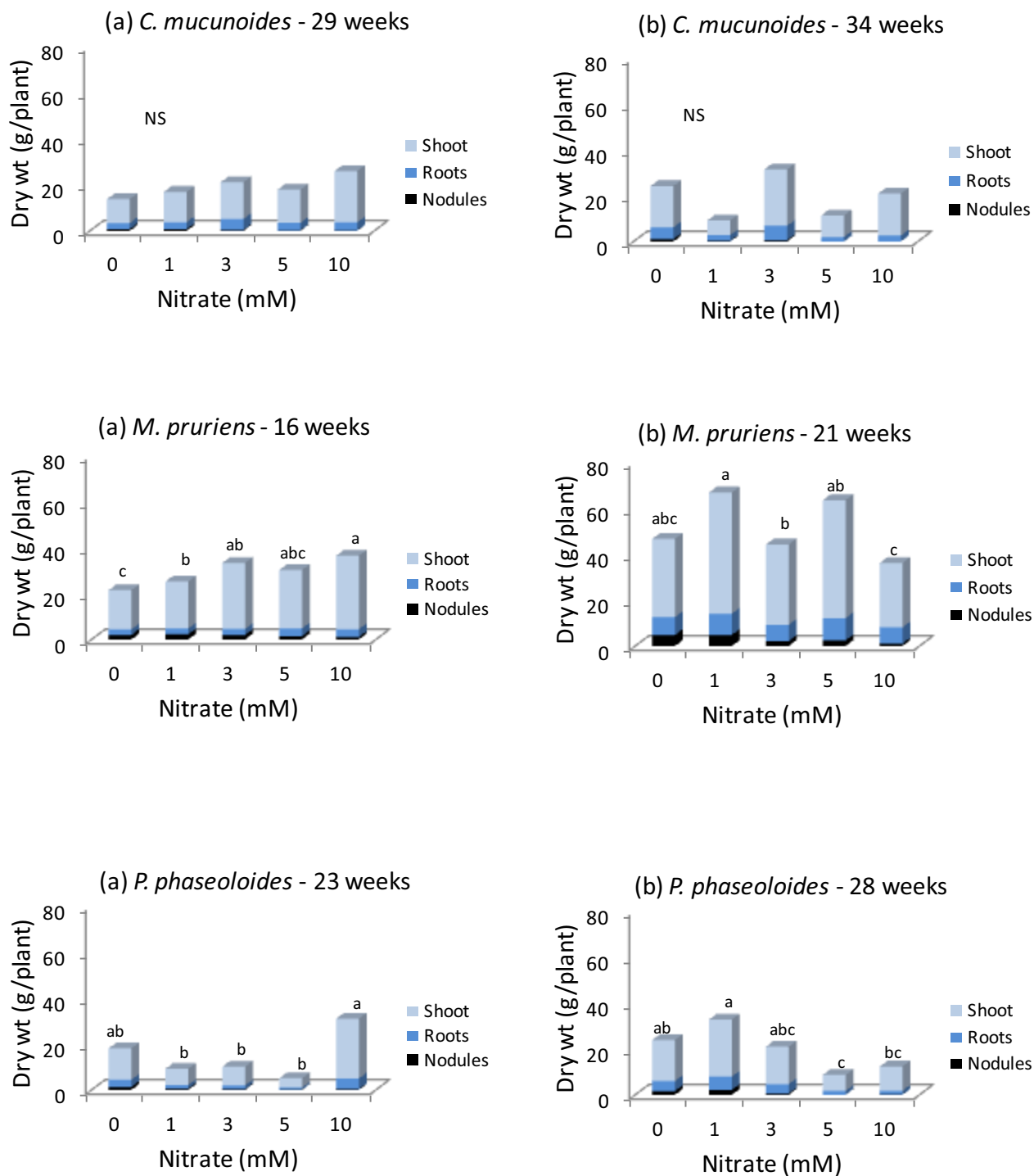
Figure 3.4: Effect of nitrate treatments on mean plant dry weights (including shoot, root and nodules) in the (a) first and (b) second harvests for: C. mucunoides, M. pruriens and P. phaseoloides. Letters indicate the lsd's at P=0.05 and bars with similar letters are not significantly different from each other.

3.3.2.2 Nodule numbers/mass

3.3.2.3 The nitrate treatments did not have a statistically significant effect on nodule numbers in C. mucunoides in either harvest, although there was a distinctive decline from zero N treatment to 10 mM nitrate (Figure 3.5; Correlation between nodule number/mass and %Ndfa

A correlation analysis (Figure 3.7) carried out between total nodule numbers and the %Ndfa obtained from ^{15}N analysis showed that the two variables are highly correlated in *C. mucunoides* (harvest 1: R=0.84, harvest 2; R=0.87), and also in *P. phaseoloides* (harvest 1: R=0.92, harvest 2: R=0.73). The relationship was poorly correlated for *M. pruriens* (harvest 1: R=0.06, harvest 2: R=0.46). Nodule mass of *M. pruriens* was used instead and there was a high correlation (R=0.81) between nodule mass and %Ndfa in harvest 1 whilst harvest 2 (R=0.03) showed a poor correlation (Appendix 5).

3.3.2.4 Plant nitrogen

3.3.2.4.1 Nitrogen concentration (%N)

Calopogonium. mucunoides nodules had a mean N concentration (%) of 5.1 and 5.7% at harvest 1 and 2 respectively (Table 3.3). There were no significant differences in nodule N concentration between each nitrate treatment at both harvests. *C. mucunoides* root N concentrations for the 5 and 10 mM nitrate treatments were significantly higher than for 0, 1 and 3 mM nitrate treatments at the first harvest, and at the second harvest root N concentration for the 10 mM nitrate treatment was significantly higher than the 0, 1 and 3 mM nitrate treatments. At both harvests *C. mucunoides* shoot N concentrations for the 5 and 10 mM nitrate treatments were significantly higher than those for the 0, 1 and 3 mM nitrate treatments.

Mean N concentration for nodules of M. pruriens were 5.5 and 4.2 % at harvests 1 and 2 respectively. There were also no significant differences in nodule N concentration between nitrate treatments in both harvests, as for *C. mucunoides* (Table 3.3). *Mucuna pruriens* root N concentration was significantly higher in the 10 mM nitrate treatment compared to all other treatments in both harvests. In the second harvest, the root N concentration was also significantly higher in 5 mM than 0, 1 and 3 mM nitrates. Shoot N concentration in the first harvest of *M. pruriens* was significantly lower at 5 mM nitrate than the other treatments. In the second harvest, the shoot N in 10 mM nitrate was significantly higher than all the

treatments, and shoot N concentration in 3 and 5 mM nitrates were also higher than the 0 and 1 mM nitrate.

Plate 2). The grand mean of total nodules in *C. mucunoides* in harvest 2 (437) was higher than in harvest 1 (311) but they were not significantly different from each other (Appendix 4).

Mucuna pruriens nodule number at harvest 1 was highest in 3 mM nitrate (352), which was significantly higher (P 0.038) than in zero (146), 1 (170), 5 (150) or 10 (93) mM nitrate treatments (Figure 3.5). The nodule number at harvest 2 was highest in the zero nitrate (232) treatment which was significantly higher than 10 mM nitrate (64). Nodule number in zero, 1, 3 and 5 mM nitrate treatments were not significantly different from each other. The mean number of nodules for *M. pruriens* in harvest 1 (182) was not significantly different to that in harvest 2 (162) (Appendix 4).

The highest nodule number for *P. phaseoloides* in harvest 1was 351 in the zero nitrate, and that was significantly higher than all other treatments Figure 3.5. Nodule number in 1 mM nitrate (168) was also significantly higher than in 5 mM nitrate (18) but not significantly different from 3 (71) and 10 (69) mM nitrates. Nodule number for *P. phaseoloides* in the second harvest was highest at 1 mM nitrate (522) and was significantly higher than the other treatments. Nodule number in the zero treatment (303) was also significantly higher than 5 (20) and 10 (17) mM nitrate. The nodule number in 1 mM nitrate in harvest 2 (522) was significantly higher than harvest 1(168). The mean number of nodules in all nitrate treatments in harvest 1 (135) was not significantly different (P=0.065) to harvest 2 (207) (Appendix 4).

When regressing the mean nodule mass (from two harvests) instead of nodule number with the percentage of N_2 fixed (%Ndfa) for the three legume species (Figure 3.6), there were high correlations for *M. pruriens* (R^2=0.88) and *C. mucunoides* (R^2=0.87) while *P. phaseoloides* showed a weak correlation (R^2=0.40).
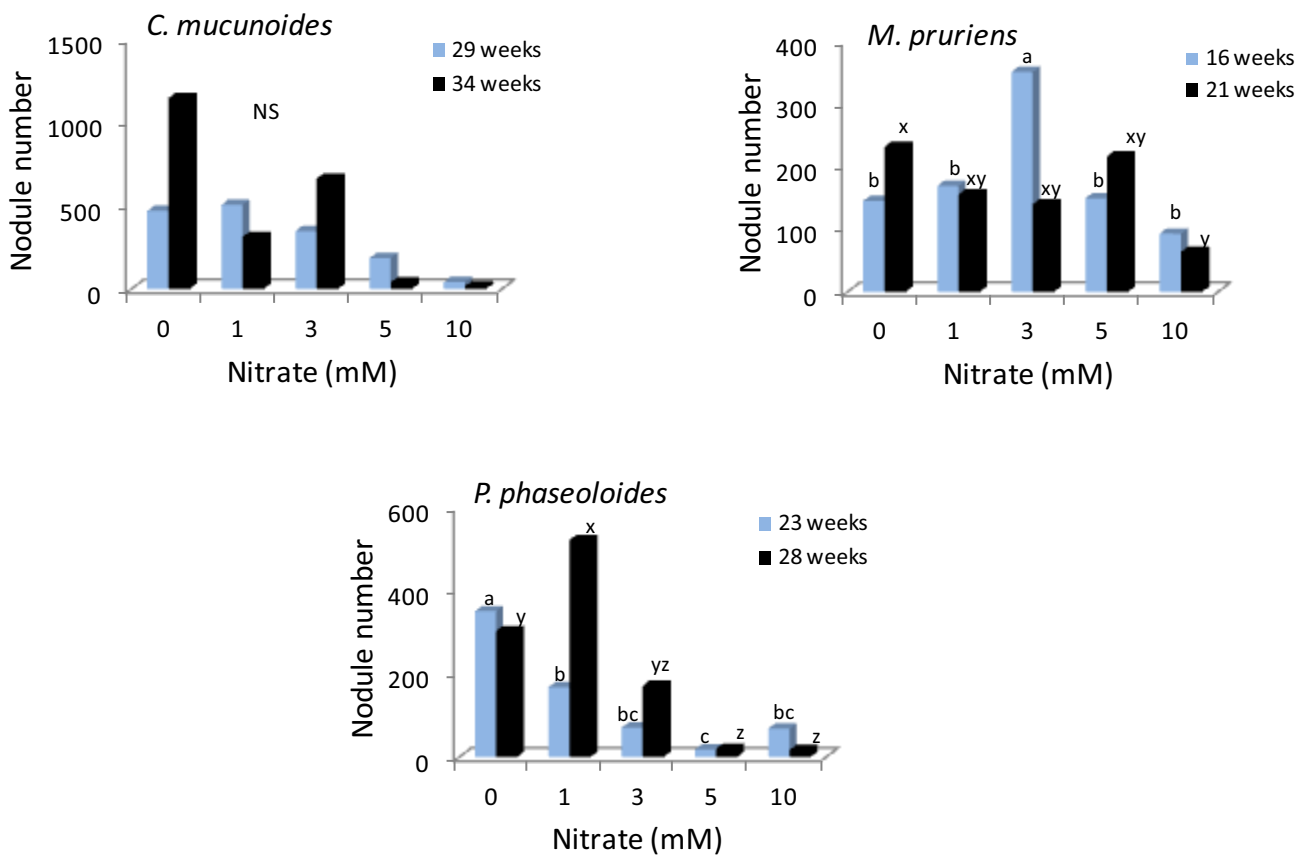
Figure 3.5: Mean number of nodules per plant at two harvests for C. mucunoides, M. pruriens and P. phaseoloides fed different concentrations of nitrate. Letters indicate the lsd's at P=0.05 and bars with similar letters are not significantly different from each other.
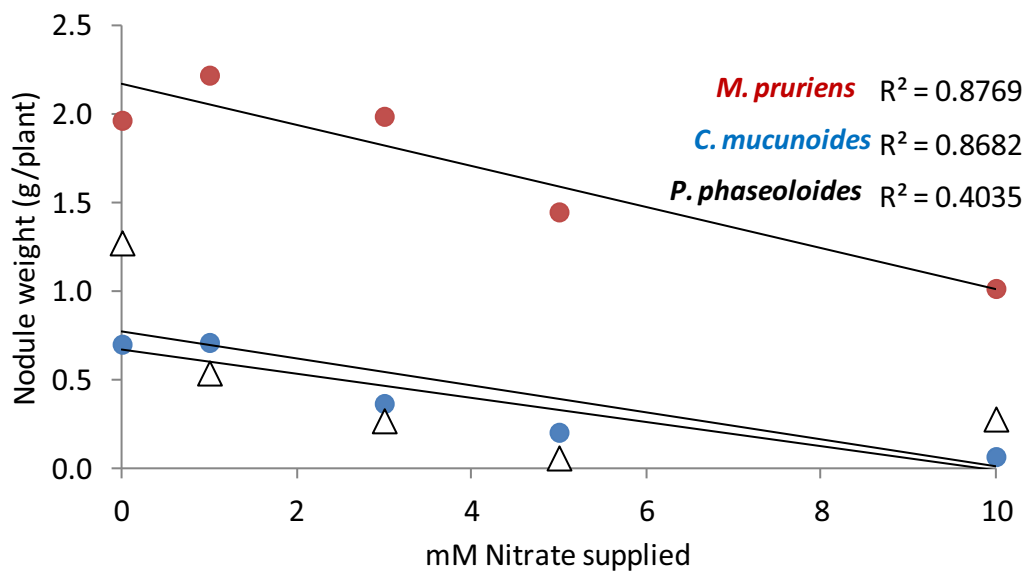
Figure 3.6: Regression and correlation of mean nodule mass (g/plant) of C. mucunoides, P. phaseoloides and M. pruriens from first harvest with applied nitrate.

3.3.2.5   Correlation between nodule number/mass and %Ndfa

A correlation analysis (Figure 3.7) carried out between total nodule numbers and the %Ndfa obtained from ^{15}N analysis showed that the two variables are highly correlated in *C. mucunoides* (harvest 1: R=0.84, harvest 2; R=0.87), and also in *P. phaseoloides* (harvest 1: R=0.92, harvest 2: R=0.73). The relationship was poorly correlated for *M. pruriens* (harvest 1: R=0.06, harvest 2: R=0.46). Nodule mass of *M. pruriens* was used instead and there was a high correlation (R=0.81) between nodule mass and %Ndfa in harvest 1 whilst harvest 2 (R=0.03) showed a poor correlation (Appendix 5).

3.3.2.6   Plant nitrogen

3.3.2.6.1   Nitrogen concentration (%N)

Calopogonium. mucunoides nodules had a mean N concentration (%) of 5.1 and 5.7% at harvest 1 and 2 respectively (Table 3.3). There were no significant differences in nodule N concentration between each nitrate treatment at both harvests. *C. mucunoides* root N concentrations for the 5 and 10 mM nitrate treatments were significantly higher than for 0, 1 and 3 mM nitrate treatments at the first harvest, and at the second harvest root N concentration for the 10 mM nitrate treatment was significantly higher than the 0, 1 and 3 mM nitrate treatments. At both harvests *C. mucunoides* shoot N concentrations for the 5 and 10 mM nitrate treatments were significantly higher than those for the 0, 1 and 3 mM nitrate treatments.

Mean N concentration for nodules of M. pruriens were 5.5 and 4.2 % at harvests 1 and 2 respectively. There were also no significant differences in nodule N concentration between nitrate treatments in both harvests, as for *C. mucunoides* (Table 3.3). *Mucuna pruriens* root N concentration was significantly higher in the 10 mM nitrate treatment compared to all other treatments in both harvests. In the second harvest, the root N concentration was also significantly higher in 5 mM than 0, 1 and 3 mM nitrates. Shoot N concentration in the first harvest of *M. pruriens* was significantly lower at 5 mM nitrate than the other treatments. In the second harvest, the shoot N in 10 mM nitrate was significantly higher than all the treatments, and shoot N concentration in 3 and 5 mM nitrates were also higher than the 0 and 1 mM nitrate.

Plate 2: Nodulated roots of C. mucunoides grown in (a) 0mM and (b) 10 mM nitrate – showing poor nodulation at high nitrate

(a)

(b)
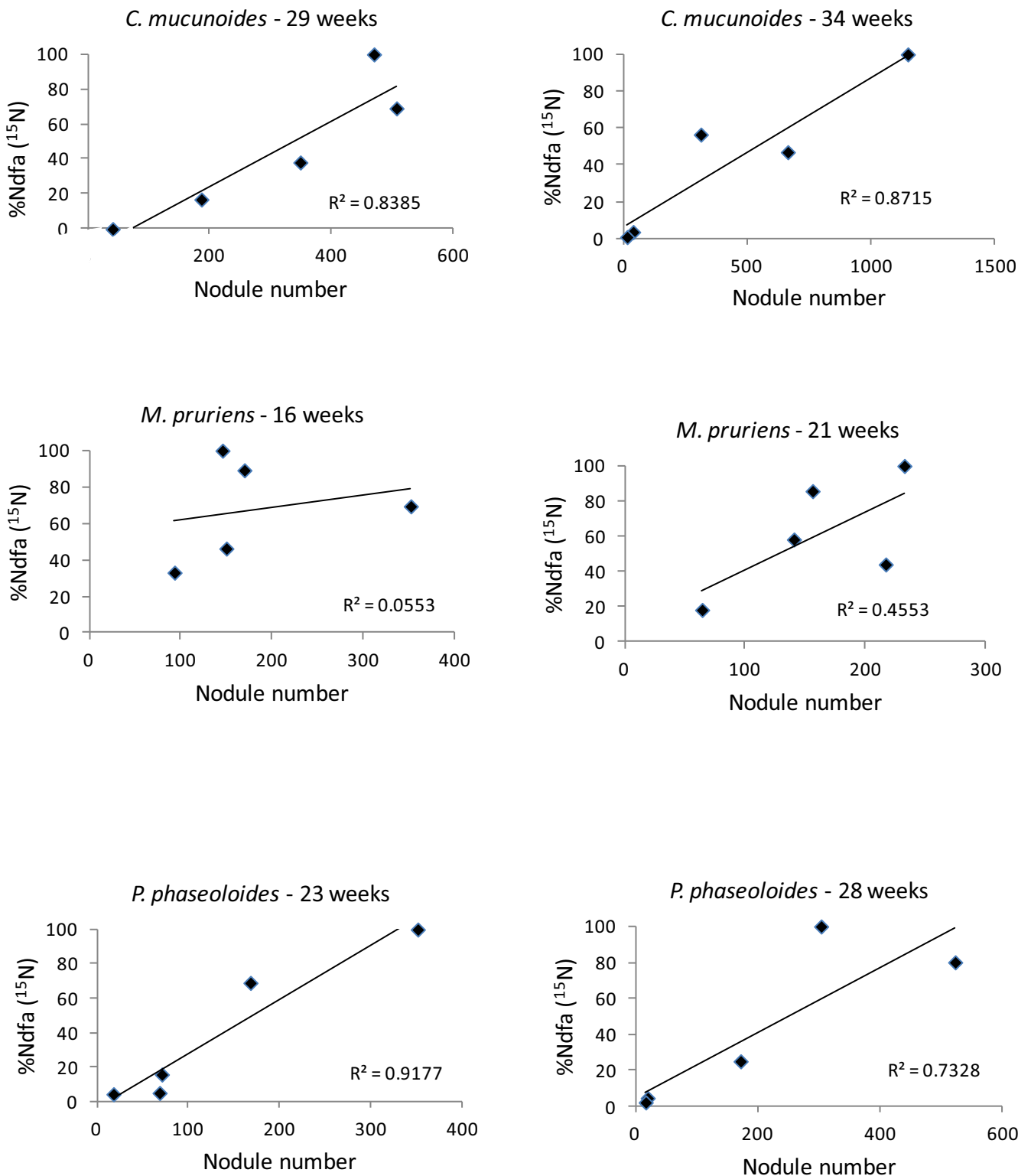
Figure 3.7: Correlations of nodule number per plant in each nitrate treatment with the %Ndfa derived from ^{15}N analysis for a) first and, b) second harvest of C. mucunoides, M. pruriens and P. phaseoloides.

There were no significant differences in the *M. pruriens* total plant N concentration between the different nitrate treatments in the first harvest. Total plant N concentration at the second harvest was highest in the 10 mM nitrate treatment and significantly different to the other treatments. *M. pruriens* plant N concentration in 3 and 5 mM nitrate treatments were also significantly higher than in the zero and 1 mM nitrate treatments.

There were no significant differences in nodule N concentration between the different nitrate treatments at both harvests for *P. phaseoloides*, similar to the results for *C. mucunoides* and M. pruriens (Table 3.3). *Pueraria. phaseoloides* root N concentration was significantly higher for the 5 mM nitrate treatment (harvest 1) and 10 mM nitrate treatment (harvest 2) than the other nitrate treatments. Root N concentration at 5 mM nitrate in the second harvest was also significantly higher than 0, 1 and 3 mM nitrates. *P. phaseoloides* shoot N concentration at the first harvest was highest (5.4%) for the 5 mM nitrate treatment, which was significantly different to all the other nitrate treatments. Shoot N concentration at the second harvest was significantly higher in the 10 mM nitrate (4.8%) treatment than the other treatments. Plant total N concentration for *P. phaseoloides* in the first harvest was significantly higher in the 5 mM nitrate than the other treatments, and at 10 mM nitrate, was significantly different to zero nitrate treatment. In the second harvest, *P. phaseoloides* plant total N concentration was significantly higher in the 10 mM nitrate than 5 mM, and 5 mM nitrate plant N concentration was significantly higher than 0, 1 and 3 mM nitrates.
Overall, across nitrate treatments and species it can be seen that nodule N concentration declined with increasing nitrate, but shoot and root N concentration tended to increase, especially in the first harvest.

3.3.2.6.2   Total N

There were no significant differences in the plant total N (Figure 3.8) or in nodules, root or shoot of *C. mucunoides* between the nitrate treatments at either harvest (Appendix 7). Nitrate treatments had a significant effect on total N in nodules, roots, shoot and total plant in *M. pruriens* between in both the first and the second harvests. In the first harvest, total N in the nodules was significantly higher in zero (0.12 g/plant) and 1(0.12 g/plant) mM nitrate than in 5 (0.08) and 10 (0.05) mM nitrates (P=0.009) (Appendix 7).

Table 3.3: Concentration of N (%N) in nodules, root, shoot at a) harvest 1 and b) harvest 2 for C. mucunoides, M. pruriens and P. phaseoloides given different amounts of nitrate.

a)

Nitrate (mM)	C. mucunoides			M. pruriens			P. phaseoloides		
	Nodules	Roots	Shoot	Nodules	Roots	Shoot	Nodules	Roots	Shoot
0	5.6	1.9	2.8	6.0	2.4	2.9	4.5	1.8	3.0
1	4.6	2.1	3.4	5.4	2.3	3.0	4.1	2.6	3.4
3	5.5	2.2	3.4	5.7	2.4	2.6	4.4	3.4	3.4
5	5.8	2.7	4.4	5.5	2.6	2.0	3.9	4.2	5.4
10	4.0	2.8	4.7	4.9	3.1	2.8	3.8	3.2	3.8
	NS	$P=0.002$	$P<0.001$	NS	$P=0.004$	$P<0.008$	NS	$P<0.001$	$P<0.001$
Grand mean	5.1	2.3	3.7	5.5	2.6	2.6	4.2	3.0	3.8
$LSD_{0.05}$		0.4	0.8		0.4	0.5		0.7	1.0
cv%		11.7	14.3		9.5	12.6		14.4	16.5

b)

Nitrate (mM)	C. mucunoides			M. pruriens			P. phaseoloides		
	Nodules	Roots	Shoot	Nodules	Roots	Shoot	Nodules	Roots	Shoot
0	6.5	2.0	2.4	4.2	1.8	1.3	4.2	1.9	2.6
1	6.3	2.1	2.1	3.9	1.7	1.4	4.1	1.8	2.4
3	4.9	2.3	2.9	4.4	2.2	1.9	4.3	1.9	2.7
5	5.7	2.8	4.7	4.5	2.6	2.3	3.6	2.4	4.1
10	4.8	3.2	4.7	4.2	3.5	3.2	4.2	2.9	4.8
	NS	$P=0.004$	$P<0.001$	NS	$P<0.001$	$P<0.001$	NS	$P=0.003$	$P<0.001$
Grand mean	5.7	2.5	3.4	4.2	2.4	2.0	4.1	2.2	3.3
$LSD_{0.05}$		0.7	0.8		0.4	0.5		0.5	0.6
cv%		17.3	16.2		11.2	16.4		16.4	11.9

Total N in the roots of *M. pruriens* was significantly higher in the 10 mM nitrate (0.1 g/plant) than 0, 1 and 3 mM nitrates (0.06 respectively). *M. pruriens* shoot total N in 10 mM nitrate (0.88 g/plant) was significantly higher than in zero (0.49) and 5 (0.5) mM nitrates. The plant total N in zero mM nitrate (0.67 g/plant) was significantly lower than in 10 mM nitrate (1.04). In the second harvest, *M. pruriens* total N in nodules was highest in the zero (0.2 g/plant) and 1 mM nitrate (0.19), and total N in 3, 5 and 10 mM nitrates were significantly lower. Total N in roots was significantly higher in the 5 and 10 mM nitrate than in the 0, 1 and 3 mM nitrates. Total N in the shoot in zero mM nitrate (0.44 g/plant) was significantly lower than in 5 (1.16) and 10 (0.82) mM nitrate treatments. *M. pruriens* plant total N at 5 mM nitrate was significantly higher (P=0.017) than 0, 1, 3 and 10 mM nitrates.

Total N in the nodules of *P. phaseoloides* in harvest 1 was significantly higher (P=0.008) in the zero nitrate (0.06 g/plant) than in the other nitrate treatments (Appendix 7). Total N in the root, shoot and whole plant of *P. phaseoloides* in harvest 1 were significantly higher (P=0.005, P= 0.002 and P=0.003 respectively) in the highest nitrate treatment (10 mM) than 0, 1, 3 and 5 mM nitrate. In the second harvest, *P. phaseoloides* total N in the nodules in zero and 1 mM nitrate were significantly higher (P<0.001) than in 3, 5 and 10 mM nitrates. The differences in the total N in root, shoot and whole plant between the different nitrate treatments were not significant in the second harvest for *P. phaseoloides*.

Across all species and harvests there were no clear trends in total plant N response to applied nitrate, excepting for *M. pruriens* which tended to accumulate more N with higher nitrate, and similarly for *C. mucunoides* in harvest 1.

3.3.2.6.3   *Amounts of N derived from N$_2$ fixation and applied nitrate-N*

The amounts of N in the three legume cover plants derived from N$_2$ fixation or nitrate are shown in Figure 3.8. In all legume species almost the entire N in the highest nitrate treatment (10 mM) came from the applied nitrate. The amount of fixed N was highest in the zero nitrate treatment and decreased with increasing nitrate in all species.
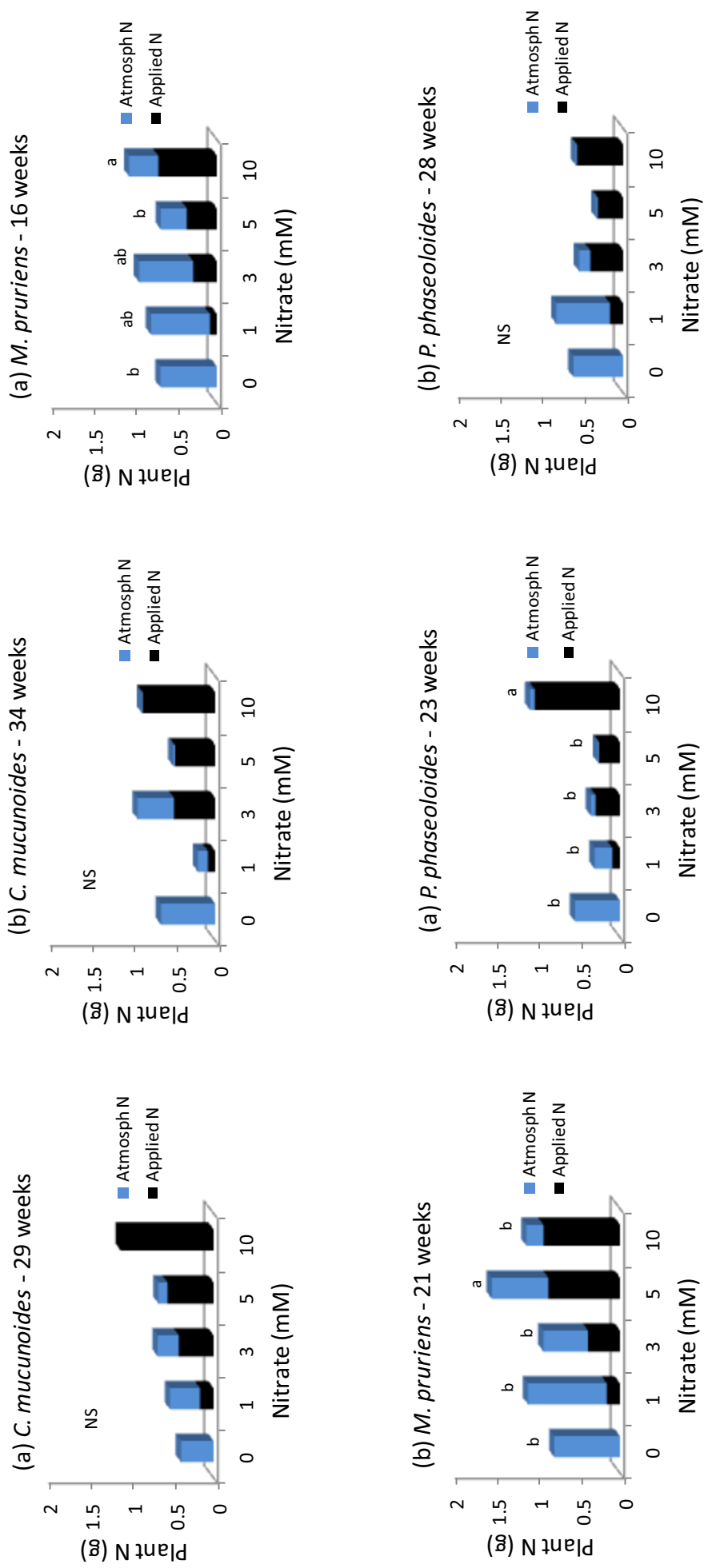
Figure 3.8: Effect of applied nitrate on the total plant N (g/plant) in each legume species with significance levels at P≤0.05. The proportions of N derived either from applied nitrate or N₂ fixation (atmospheric N) are shown as well.

3.4   Discussion

The main aim of the experiment was to use the ^{15}N isotope dilution technique to calibrate the xylem ureide technique, which was successfully achieved. In *C. mucunoides* and *P. phaseoloides*, the RU-N (%) was highly correlated (R^2=0.7 and 0.8 respectively) with the %Ndfa that was obtained from ^{15}N analyses indicating a positive relationship between active N_2 fixation and the transportation of ureide-N in the xylem. However, *M. pruriens* had a weak correlation ($R^2 = 0.4$) between N_2 fixation and transportation of fixed N as xylem ureide-N. The calibration equations: y=0.5971x + 0.7882 and y=0.5374x + 0.3806 for *C. mucunoides* and *P. phaseoloides* respectively could now be confidently used to measure N_2 fixation in these species. The slopes for *C. mucunoides* (0.5971) and *P. phaseoloides* (0.5374) are similar to those of pigeon pea (Peoples *et al.* 1989), and common bean (Unkovich *et al.* 2008) obtained from calibration studies. The calibration equation for *M. pruriens* (y=0.1311x + 0.5023) has a smaller slope due to very low ureide levels in all the nitrate treatments and a very high %Ndfa. This slope value is close to that obtained by Herridge et al (1996) for the tree legume species *Codariocalyx gyroides*. These calibration equations provide a basis for further work on *C. mucunoides*, *M. pruriens* and *P. phaseoloides* or related species. However, further investigation is required since the current study is inconclusive about the ability of *M. pruriens* to transport ureides as direct by-products of N_2 fixation. Also, there is no other work done on the calibration of xylem sap ureide for *C. mucunoides*, *M. pruriens* and *P. phaseoloides* so comparisons are limited. The stem RU-N and %Ndfa discussed here are the means of two harvests and these combined values are used because the calibrated equation will be applied to different ages of legumes in the field. Secondly, the combined values gave a stronger correlation between the stem RU-N and %Ndfa, and gave more confidence for its application.

M. pruriens, on the other hand, had very low stem RU-N in both harvests (mean of <9%) which makes it difficult to relate the RU-N calibration of *M. pruriens* to N_2 fixation in a field situation where stem RU-N is much higher than this mean, for example, in the PNG field survey (Chapter 4) although the mean RU-N (>20%) there was measured in a single location. The low glasshouse RU-N (mean of 5.3%) in *M. pruriens* raises doubts as to the validity of using stem RU-N as a measure of N_2 fixation in *M. pruriens*. However, although it may be a relatively poor ureide-transporter, there was still a significant decrease in stem RU-N in response to high nitrate in *M. pruriens* which suggests that *M. pruriens* does transport ureide as a product of N_2 fixation. This, and the slightly higher (>20%) RU-N

measured in the field survey (Chapter 4) holds some promise that it could be possible to utilise stem RU-N as a measure of N_2 fixation in *M. pruriens* and warrants further investigation. It would be beneficial to undertake a further calibration experiment for *M. pruriens* under more ideal growing conditions to ensure optimal ureide levels, and hence better assess the ureide technique for this legume species.

The second aim of the study was to investigate the response of the three legumes to nitrate. At higher nitrate concentrations *M. pruriens* and *P. phaseoloides* had corresponding increases in plant total N content, while N_2 fixation was significantly reduced. The plant total N response for *C. mucunoides* was less defined which was attributed to overall slow growth. It was also interesting to note that total N of nodules for *M. pruriens* and *P. phaseoloides* were significantly reduced at higher nitrate concentration in both harvests, which could directly relate to reduced N_2 fixation, but the mechanism is not well understood. The negative effect of nitrate on N_2 fixation in legumes has been reported by many (Herridge 1982; Herridge and Peoples 1990; Herridge and Peoples 2002a; Herridge and Peoples 2002b; McClure *et al.* 1980; Pate *et al.* 1980; Pate and Dart 1961; Streeter 1982; Streeter 1985; Streeter and Wong 1988). *M. pruriens* had a higher plant biomass compared to *C. mucunoides* or *P. phaseoloides* whose growths were relatively similar in both harvests. *M. pruriens* is naturally a fast growing plant with larger plant biomass compared to *C. mucunoides* or *P. phaseoloides* (Giller 2001b) which means it is better at nutrient acquisition as well. While this study showed that nitrate significantly affected N_2 fixation in all three species, Carmagos and Sodek (2010) reported that nodule number was reduced but nodule growth (nodule mass/plant) and N_2 fixation in *C. mucunoides* was unaffected by nitrate in a glasshouse experiment conducted in Brazil. The two experiments were relatively similar in that ureide concentration in xylem sap was measured after the application of a zero and a high nitrate treatment (15 mM nitrate in the Brazil experiment). However, the Brazil experiment ran for 45 days before the xylem sap was analysed while the current experiment went longer which may have allowed enough time for the nitrate effect on N_2 fixation to be observed. Furthermore, the %Ndfa measured by the ^{15}N isotope dilution technique in the current experiment also strongly supported the effect of nitrate on reducing N_2 fixation in *C. mucunoides*.

Using ^{15}N isotope dilution, the mean of two harvests showed that *M. pruriens* was still fixing as much as 26% of its N when fed with 10 mM nitrate while *C. mucunoides* and *P. phaseoloides* were fixing <4%. *M. pruriens* may have a higher tolerance level to nitrate compared to *C. mucunoides* and *P. phaseoloides*, or the N uptake was being incorporated into the rather fast growth of *M. pruriens* so that the nitrate did not reside long enough in the growth media to have the reducing effect on N$_2$ fixation. *M. pruriens* could become the choice legume cover plant in agricultural systems where there is high usage of mineral N fertilizers, which could not be easily substituted or reduced, such as in the oil palm system, since it (*M. pruriens*) could withstand higher nitrate concentration while maintaining N$_2$ fixation. Due to its rapid growth and larger biomass, *M. pruriens* could accumulate higher plant N at a time, although a recent field survey in PNG oil palm plantations showed that an excellent *P. phaseoloides* growth had a comparable biomass (443 g/m^2) to *M. pruriens* (408 g/m^2). *M. pruriens* evidently grew better than both *P. phaseoloides* and *C. mucunoides* in four different experimental sites in Cote d'Ivoire over 6 months of growth (Becker and Johnson 1999). The experiment studied N accumulation and the weed suppression capabilities of 54 legume accessions. The four sites: derived savannah, monomodal forest, guinea savannah and bimodal forest had been sown to rice-cotton rotations, rice-soybean rotations, sole crop of rice-forest fallow and rice-cassava-forest fallow rotations respectively, with soils varying in soil pH and available P levels. In all four sites *M. pruriens* maintained a higher legume biomass (range 0.98-12.74 t/ha), in three of which legume biomasses were significantly higher (LSD$_{0.05}$) than *P. phaseoloides* (range 0.51-4.93) or *C. mucunoides* (range 0.17-4.55) (Becker and Johnson 1999).

It was interesting to note in the current glasshouse study that the nodule N concentration (%N) was not affected by the applied nitrate in any legume species in both harvests. Only the total N was significantly reduced. The latter being a function of nodule mass and N concentration, nodule mass could be affected anytime by the legume growth, root proliferation and subsequent increase in nodule number and/or size. There was no response in nodule total N, though, or any other biomass measurements in *C. mucunoides* which is attributed to the very poor growth observed in all the treatments possibly due to toxicity. Since the *C. mucunoides* biomass is affected, much of the other responses were masked. Even so, the unchanging nodule N concentrations signify that the source of N within nodules came directly from N$_2$ fixation in all three legume species and does not change irrespective of the nitrate concentration in the surrounding medium. Nodulation was

directly related to N_2 fixation as observed in this study, either in the total nodule number (best shown for *P. phaseoloides*) or the nodule mass (as shown for *C. mucunoides* and *M. pruriens*), and just like other parameters of N_2 fixation measured, nodulation was significantly affected by nitrate. Visual observations of the nodule types for the three legumes (Plate 3) showed that *M. pruriens* had indeterminate nodules while *P. phaseoloides* and *C. mucunoides* had determinate ones. Legumes with indeterminate nodules have a persistent meristem from which new nodules are initiated and extended whereas determinate nodules have a defined lifespan and are usually shed after senescence (Hansen 1994). The strong correlations between the total number of nodules (or the nodule mass) and the %Ndfa for each species could be refined further in a repeated calibration experiment so that nodule numbers/masses could be used in a simple model to suggest correlated levels of %Ndfa for *C. mucunoides*, *P. phaseoloides* and *M. pruriens*.

The *P. phaseoloides* shoot N concentrations (range 2.4-5.4%) in the current study have some values that were higher than some of the *P. phaseoloides* values (range 2.4-3.8%) collated from several different field studies (Gil *et al.* 1997; Suvannang *et al.* 2010; Tian and Kang 1998; Viera-Vargas *et al.* 1995; Zaharah *et al.* 1986). Typically plant N concentration will increase with the nitrate supply in the growth media (Barker and Bryson 2007). The higher values in the current glasshouse study were obtained in the higher nitrate treatments (10 mM). All the *P. phaseoloides* collated values (range 2.4-3.8%) discussed here were very close to the mean shoot N concentration (2.69) recorded for field grown legume crops and pastures in Australia as well (Unkovich *et al.* 2010a) showing what could be expected from field legumes N concentrations. Barker and Bryson (2007) listed <3.6, <3.8 and <2.2% dry mass of leaves to be the low diagnostic ranges for grain legumes, forage legumes and herbaceous perennials (such as *C. mucunoides*, *P. phaseoloides* and *M. pruriens*) respectively. If so, the values recorded by Unkovich, Baldock and Peoples (2010) may be on the low side, where symptoms of deficiency would be showing (Barker and Bryson 2007). On the other hand, the range discussed above (2.4-3.8%) from other *P. phaseoloides* studies were within the sufficient range. The current glasshouse values that were >4.0 % were in the high range (Barker and Bryson 2007) as were observed in the *C. mucunoides* and *P. phaseoloides* when supplied with 5 and 10 mM nitrates. Both *C. mucunoides* and *P. phaseoloides* appeared to have extremely high shoot N concentration compared to *M. pruriens* in this glasshouse study and this is possibly because these species had much slower growth than *M. pruriens* and excess N accumulated in toxic amounts.

Indeed, there were curled leaves and stunted growth characteristics that accompanied *P. phaseoloides* and especially *C. mucunoides* throughout the glasshouse study which may support this assumption.

A few other pot trial studies have been carried out that relate to the same legume species reported here. A study worth looking at for legume growth comparison in pot trials was conducted by Tian and Kang (1998) in Ibadan, south-western Nigeria on the effect of soil fertility and fertilizer application on different legumes' biomass and chemical compositions. Twelve legume plants including *M. pruriens* and *P. phaseoloides* were grown and harvested after 18 weeks on two soil types (high and low fertility) either with or without N, P and K fertilizers which were applied at a rate of 22 mg/kg soil each. *M. pruriens* had a plant dry weight range of 22 to 69 g/pot while *P. phaseoloides* had 8 to 48 g/pot with the highest values obtained in the high fertility soil+fertilized pots. These dry weights are for 2 plants per pot, so technically, half of the dry weights would resemble the current glasshouse study of 1 plant per pot. The mean dry weight then (from two harvests) of *M. pruriens* (40.5 g/pot) is related to the values obtained in the high fertility soil+fertilized treatments, while *P. phaseoloides* had a lower dry weight (17.2 g/pot) which showed that it could have grown better in the current glasshouse study.

Generally the glasshouse environment in Adelaide was not ideal for growing tropical legumes and in particular temperatures may have been too low for optimal growth, which caused the more slow-growing species (*C. mucunoides* and *P. phaseoloides*) to develop poorly. However, apart from one water stress episode during an unexpected hot period that caused substantial leaf drop, the *M. pruriens* growth was excellent. If growth had been optimal in all species, the uptake and partitioning of the fed nitrate into biomass production may have had a more positive effect, especially on *C. mucunoides* where response to nitrate in the measured parameters, except for dependence on N_2 fixation, was not strongly defined. To obtain more reliable observations on all three species, this experiment could be repeated in a warmer environment so that the responses in *C. mucunoides* and *P. phaseoloides* could be confirmed, and also the stem RU-N in *M. pruriens* could be further studied. Regardless of the difficulties discussed above, the ureide technique was successfully calibrated for *C. mucunoides* and *P. phaseoloides* and could now be applied in the estimation of N_2 fixation by cover legume species in the field in PNG.

Plate 3: Determinate nodules of (a) Pueraria Phaseoloides and (b) Calopogonium mucunoides, and(c) indeterminate nodules of Mucuna pruriens

(a)

(b)

(c)

4 Estimating nitrogen fixation by *Calopogonium caeruleum, Pueraria phaseoloides* and *Mucuna pruriens* under different aged oil palm plantations using the ureide technique

4.1 Introduction

Biological N_2 fixation by different legume species has played a major role in many agricultural systems in the world as highlighted in the literature review. Due to their fast growth and creeping nature, some legumes are used as cover plants under plantation crops for the purpose of weed suppression, soil erosion control and importantly as N_2 fixers (Fairhurst and Hardter 2003). Legumes such as *C. caeruleum*, *C. mucunoides*, *P. phaseoloides*, *M. pruriens* and *C. pubescens* are common legume cover plants used under rubber and oil palm plantations in the tropics (Giller and Wilson 1991). Except for *C. pubescens*, all of these legume cover plants have been used for a long time or recently introduced in the oil palm plantations in Papua New Guinea. There have been no assessments though carried out on how much N_2 these legume cover plants are fixing and contributing to the oil palm plantations, which becomes the focus of this study.

In order to measure N_2 fixation, two important measurements are required: a) a measure of the percentage of N_2 fixed by legumes (%Ndfa) and b) the total N in the legume biomass. Many techniques are available for assessing N_2 fixation (Unkovich *et al.* 2008). For this exercise the ureide technique will be used to measure the percentage of N_2 fixed by legumes, and a destructive sampling will be carried out on field legumes to estimate legume biomass production and total N. Since the ureide technique provides an indirect method for assessing N fixation, the glasshouse study was carried out for the purpose of calibrating this technique against a more reliable technique, the ^{15}N isotope dilution (Herridge and Peoples 1990) before applying it to measure N_2 fixation. The resulting calibrated equation from correlating and regressing the percentages of N fixed (from ureide and ^{15}N isotope dilution) is used in the following study to measure N_2 fixation by *P. phaseoloides*, *C. mucunoides, P. phaseoloides* and *M. pruriens* under PNG oil palm plantations. Due to the fact that these legumes occur under different ages of oil palm plantations, observation is also made on the N_2 fixing performance of legumes covers under different oil palm ages.

4.2   Materials and methods

4.2.1   Background

The survey was carried out in the New Britain Palm Oil Limited (NBPOL) plantations near Kimbe, West New Britain Province in Papua New Guinea. Kimbe is located on the western part of New Britain, the largest island in Papua New Guinea (Figure 4.1). Sampling was carried out in three groups of plantations: Mosa, Kapiura and Numundo (Figure 4.2) with oil palm ages ranging from 2 to 25 years.

Figure 4.1: Map of Papua New Guinea with Kimbe circled with dotted yellow line.
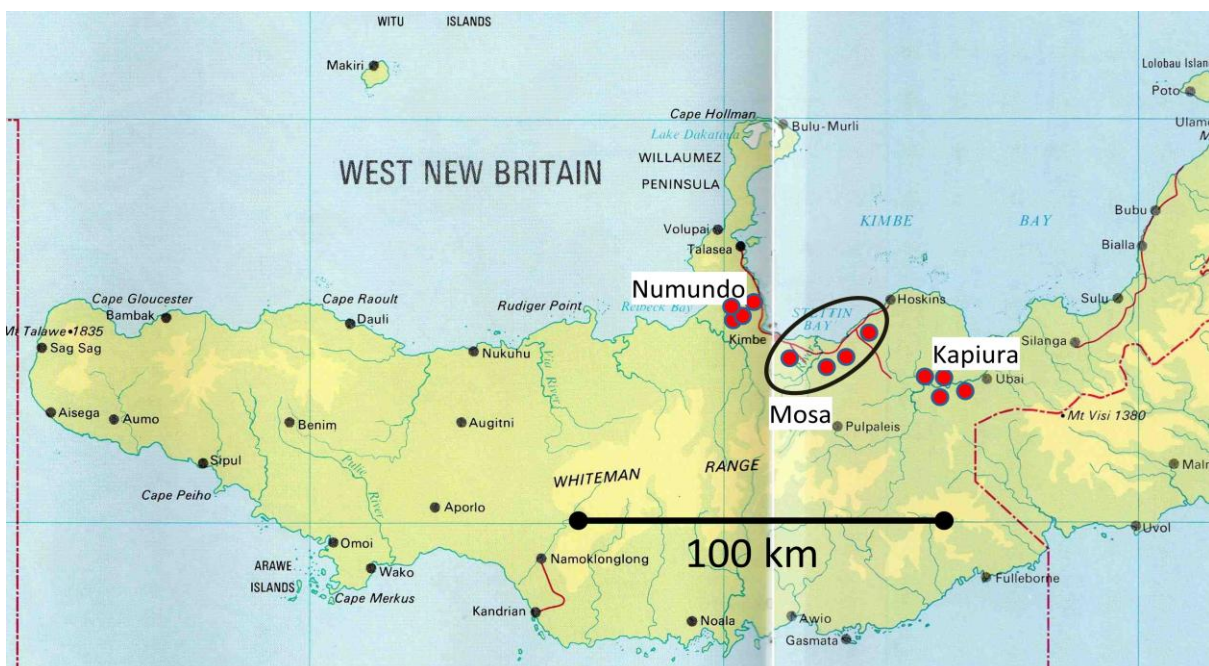
Figure 4.2: Map showing areas (in red) sampled for N_2 fixation study in three New Britain Palm Oil plantation groups in Kimbe, WNBP.

84

The climate in the study region is tropical with an average annual rainfall of 3600 mm. The wettest months are from December to March (refer Figure 4.3). The topography is hilly with mountain ranges and several volcanic peaks surrounding the cultivated areas established on the flatter ground. The soil type is of volcanic ash origin with sandy loam to clay in the top 30 cm and alternating with pumice and gravel throughout the soil profile.
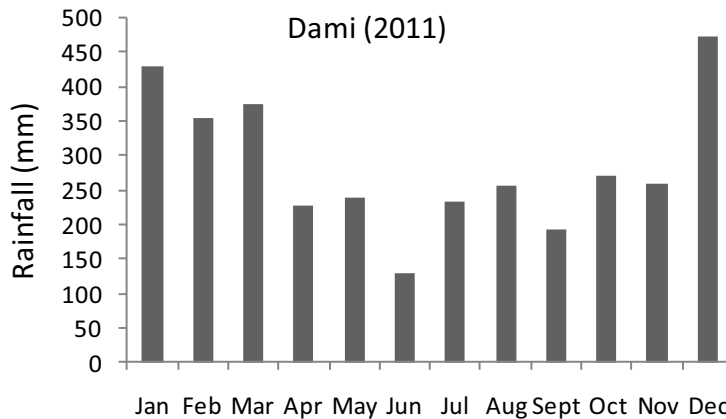
Figure 4.3: 2011 monthly rainfall data for Dami plantation in Kimbe West New Britain Province, Papua New Guinea

4.2.2   Site information and fertiliser management

Sampling was carried out 12 different oil palm field ages in three main locations (Table 4.1). Each field had an average area of 27 hectares with the planting densities ranging from 120 to 135 palms per hectare. It is important to note here about the triangular distances between palms since it influenced the way legume cover was estimated in this survey. The standard triangular distance between three adjacent palms is 9 x 9 x 9 meters in oil palm plantations (Corley and Tinker 2008). Different planting densities increased or decreased these distances slightly. A range of mineral fertilizers were applied in split applications in 2011 (Table 4.1). Fertilizer types and rates are usually recommended depending on the oil palm leaf analysis results from the previous year. Ammonium nitrate, ammonium chloride, borate, triple superphosphate, muriate of potash, potassium sulphate and magnesium sulphate (kieserite) are the types of fertilizers usually applied in PNG oil palm plantations as recommended. Fertilizer is usually broadcast everywhere under the palm (Banabas 2007)

except when the palms are immature (0-2 year old) when they are applied in the weeded circle only (Figure 4.4). Legume cover plants used are C. caeruleum, P. phaseoloides and more recently C. mucunoides. Also M. pruriens was recently planted in a few locations as a trial before being integrated as a main legume cover under oil palm. Seeds of C. caeruleum, P. phaseoloides and C. mucunoides are mixed in the recommended ratio of 2.5:3.5:1.5 kg/ha (Fairhurst and Hardter 2003) and sown in three lines between two adjacent palms in an oil palm row (Figure 4.4) For the last 5 to 6 years seeds of *P. phaseoloides* and *C. mucunoides* only were mixed and sown due to low supply of *C. caeruleum* in the West New Britain Province. *P. phaseoloides*, *C. mucunoides* and *M. pruriens* are not shade tolerant so they die out after four to five years when the oil palm canopy closes (Giller and Wilson 1991); only *C. caeruleum* thrives under older plantations.

Table 4.1: Plantation age and fertilisers applied in 2011 for the oil palm plantations in Kimbe, West New Britain Province in which N_2 fixation assessment was conducted.

Species	Location	Year	Age (years)	Fertilizers applied in 2011 (kg/palm)				
				N	P_2O_5	K_2O	MgO	B
C. caeruleum	Mosa	2008	4	1.05	0.24	1.71	0.23	0
C. caeruleum	Mosa	1999	13	1.04	0.40	1.75	0.24	0.011
C. caeruleum	Mosa	1994	18	1.37	0.57	1.75	0.16	0
C. caeruleum	Kapiura	2009	3	0.73	0.25	1.27	0.18	0.009
C. caeruleum	Kapiura	2007	5	0.87	0.28	1.74	0	0
C. caeruleum	Kapiura	2005	7	0.48	0	0	0	0
C. caeruleum	Kapiura	1987	25	0	0	0	0	0
C. caeruleum	Numundo	2003	8	0.82	0	1.00	0.15	0.010
C. caeruleum	Numundo	2004	9	0.65	0	0	0	0.010
C. caeruleum	Numundo	1996	16	0.24	0	0.40	0	0
P. phaseoloides	Mosa	2010	2	0.89	0.44	1.22	0.16	0.019
P. phaseoloides	Mosa	2008	4	1.05	0.24	1.71	0.23	0
P. phaseoloides	Kapiura	2009	3	0.73	0.25	1.27	0.18	0.009
P. phaseoloides	Kapiura	2007	5	0.87	0.28	1.74	0	0
P. phaseoloides	Numundo	1998	4	1.01	0	1.75	0	0.011
M. pruriens	Mosa	2006	6	1.04	0.40	1.51	0.24	0.011

Legume biomass (both shoot and litter), nodule scoring, legume cover estimation and soil analyses were carried out for three legume cover plants, *C. mucunoides*, *P. phaseoloides*

and *M. pruriens* in Mosa and Kapiura. Not all measurements were made at Numundo because of limited time factor but sampling of legumes stems for xylem ureide analyses and percentage of legume cover estimations were done in all three plantation groups. Due to differing management strategies and legume growth not all species occurred in all the plantations. *M. pruriens* was only found at one location (Mosa young planting) since it was only recently introduced to test its performance before being integrated as a legume cover plant in the oil palm system.
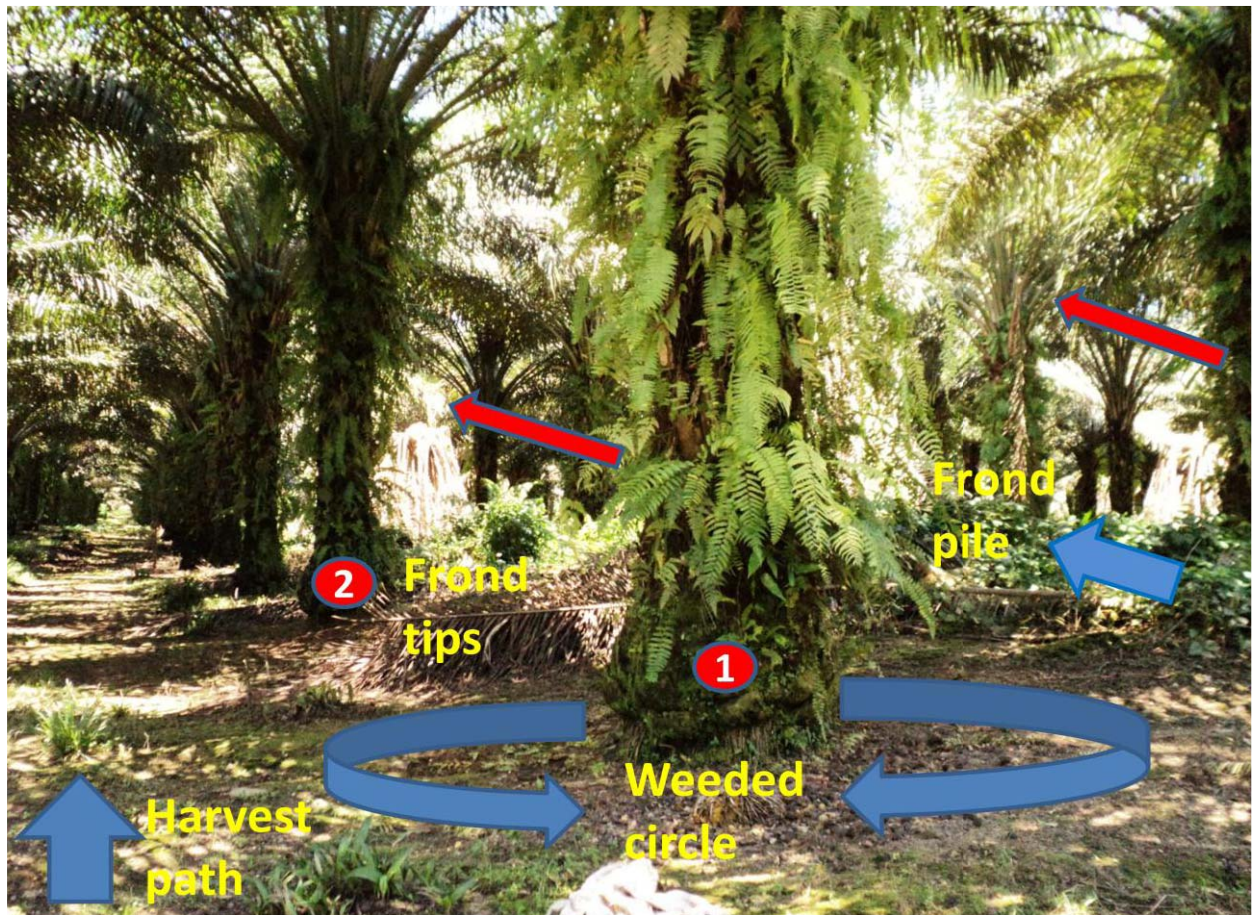
Figure 4.4: The 'zones' under a 7 years old oil palm plantation in PNG.

Note: The harvest path and the frond pile alternates between every row (red arrow) of oil palm. Frond tips is the zone between two palms where the tips of oil palm leaves (or fronds) are usually placed while the weeded circle is a 1 meter band weeded around the base of the palm. The frond pile is where the rest of the harvest fronds are accumulated.

4.2.3   Legume plant sampling, nodule scoring and soil sampling

Legumes were surveyed under palms aging from 2 to 25 year old. Ten replicate legume samplings were made in each plantation under study for the different legume cover plants, *C. mucunoides, P. phaseoloides* (Plate 4*a*)*, C. caeruleum* and *M. pruriens* where they occurred, by randomly selecting areas containing legume cover and collecting shoot biomass within a $1m^2$ quadrat (Plate 5). In all cases, the areas sampled contained single legume species. Although the purple stem of P. phaseoloides was distinctive in the young plantations (Plate 4b), due to similar features it was not easy in many cases to differentiate *C. mucunoides* from the *P phaseoloides* and in these cases material was recorded as *P. phaseoloides.* The green legume shoots were first removed, and then the litter layer (Plate 6) under the legume was then also placed into a separate bag. The fresh weight of the $1m^2$ samples were taken and then the shoot sample was chopped up into smaller sizes, then quartered and two quarters selected, mixed again, quartered again and a final quarter (1/8th) sample taken for dry weight and N determination. The fresh weight of this subsample was recorded. The same sub sampling procedure was repeated for the $1m^2$ litter samples. Sixteen (16) stem segments, around 10 cm long were collected from every quadrat and kept separately in paper bags for relative-ureide N analysis. Stem segments, shoot biomass and litter samples were oven dried at 48^oC for two days before grinding. Samples had to be irradiated to comply with quarantine regulations before analysis in Australia. Dried samples were thus subjected to gamma irradiation treatment at a dosage of 25kGray (Steritech Pty Ltd in Queensland Australia) before being sent to the University of Adelaide for relative ureide and total N analysis.

Plate 4: Pueraria phaseoloides in the two year old Mosa Plantation (a) overview of cover and (b) close up to show distinctive purple stem

(a)

(b)

*Plate 5: Sampling (a) Pueraria phaseoloides and (b) Calopogonium caeruleum with a 1m²
quadrat*

(a)

(b)

Plate 6: Litter layer in (a) Calopogonium caeruleum and (b) Pueraria phaseoloides and (c) presence of decaying nodules in the litter

(a)

(b)

(c)

Plate 7: Random scoring of nodulation on legume cover roots was undertaken in the plantations

Independent and random scoring of legume nodulation was conducted in all plantations (Plate 7) using the scoring system similar to that described by Corbin et al (1977) and illustrated in Figure 4.5. The system was adapted to the study legumes as illustrated in Table 4.2.
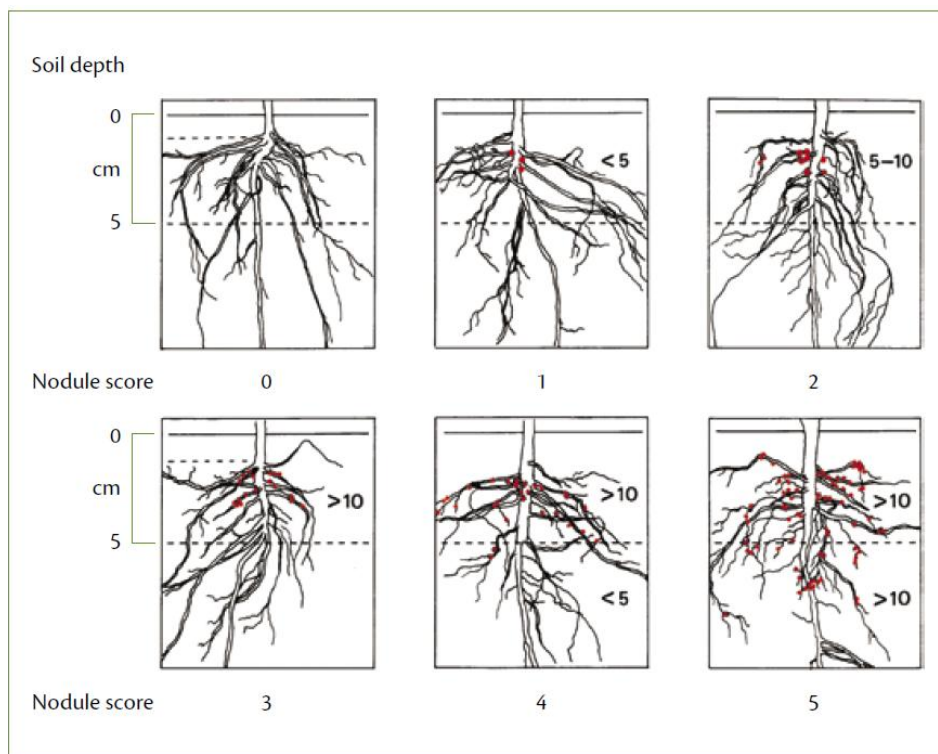
Figure 4.5: Nodule scoring system derived from Corbin et al (1977)

Table 4.2: A slightly modified version of the scoring system used by Corbin et al (1977) to assess nodulation in the legume cover under oil palm

Root depth in soil (0-5 cm)	Root depth in soil (>5 cm)	
Total number of pink/red nodules		Score
0	0	0
<5	0	1
5-10	0	2
>10	0	3
>10	<5	4
>10	>10	5

0-2 indicates poor nodulation and probably little or no N_2 fixation

2-3 represents fair nodulation; N_2 fixation may not be sufficient to supply the N demand of the crop

3-4 indicates good nodulation; good potential for N$_2$ fixation

4-5 represents excellent nodulation; excellent potential for N$_2$ fixation

Using a 150 cm auger to a depth of 20cm four soil samples were taken from every second 1m^2-quadrat of legume biomass. The four soil samples were mixed well in a bucket and about 500 grams taken for fresh weight, oven-dried at 60°C until dry and the dry weight recorded. About 100grams from the dried soil samples were then sent to the University of Adelaide for N analysis, after the irradiation treatment described above.

4.2.4   Legume cover estimation

The legumes were not spread evenly across the plantations but occurred mostly in areas between adjacent palms along a row where they would have been sown during oil palm planting. The total legume cover in each of the oil palm planting surveyed was estimated. Due to the triangular layout of adjacent palms (see section 4.2.2) in an oil palm planting, a sampling strategy was devised to enable sampling of a representative area (Figure 4.6).
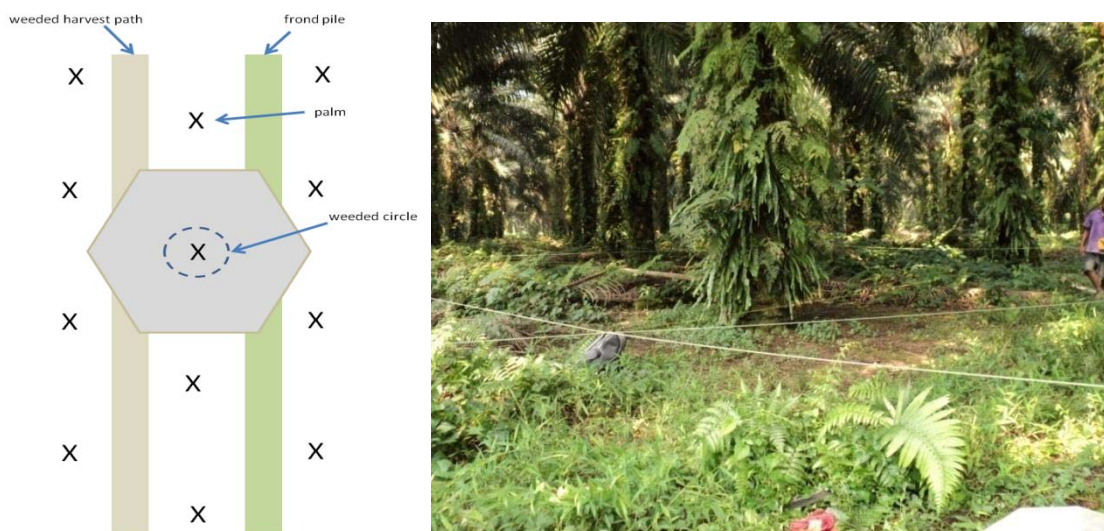
Figure 4.6: Legume cover was estimated using a (a) hexagonal area shaped out using (b) nylon ropes tied around palm bases in such a way that covered all the identified 'zones' under an oil palm plantation

A hexagonal area was constructed in such a way to proportionally represent the oil palm plantation which included the oil palm, the weeded circle, the harvest path, the frond pile separating every two rows of palm, and the frond tips (area between two palms along the same row that usually contain harvested frond tips). The hexagon was blocked out using

nylon ropes (Figure 4.6) and the dimensions of all hexagonal sides, plus two triangles and a rectangle within the hexagon was taken using a tape measure, to calculate the area of the hexagon. A one square metre quadrat was then moved from place to place to measure the total area covered by the legumes within the hexagon by placing the quadrat over the legumes only and estimating the number of square metres under legume cover within the hexagon. This was repeated ten times within each planting type, and the average area covered by legumes was estimated in percentage per area of the hexagon (% legume cover per m^2).

4.2.5   Laboratory analysis

The ground stem samples were analysed for the different sap N compounds (ureide-, nitrate- and amino-N) using the methods described in Chapter 2. Other ground plant material (shoot and litter) were sent to the Waite Analytical Lab for the analysis of total N by automatic combustion. 10g of air-dried and sieved soil was extracted in 40 mL 2M KCL for 1 hour and then filtered through a Whatman No.1 filter paper. Extracts were frozen until analysis. Aliquots of defrosted and shaken extracts were analysed for ammonium-N and nitrate-N colorimetrically (Rayment and Lyons 2011) on an Alpkem Flow Solution III autoanalyser.

4.2.6 Calculating the amount of N_2 fixed (g/m^2) in field legumes

The amounts of N_2 fixed by the different legume species were determined using the %Ndfa, legume shoot N concentration and legume biomass. By inserting the measured stem RU-N values into the calibrated equation (Figure 4.9), the %Ndfa was determined for each legume cover species. Total amount of N_2 fixed (g/m^2) by each legume species was then calculated as follows:

Amount of N_2 fixed = (%Ndfa/100) x [(legume N concentration *legume biomass)/100] *(Equation 7)*

In this case, legume N concentration was analysed separately for both shoot and litter and the same %Ndfa estimate was used to calculate the proportion of N derived from biological N_2 fixation in both legume shoot and litter.

4.3 Results

4.3.1 Shoot and litter dry matter (g/m²)

The shoot dry weights of the different legume species are presented in grams per square meter (g/m^2) of legume and then converted to kilograms per hectare (kg/ha) of plantation using the legume cover estimation. The shoot dry weights ranged from 144 g/m^2 to 443 g/m2 across all legume species and plantations (Table 4.3). Legume shoot biomass was higher in the younger oil palm plantings, while the lower shoot dry weights were found in the more mature plantings (7-25 year old), which consisted mainly of *C. caeruleum* also had a lower (176 g/m^2) shoot dry weight in a 5 year old planting in Kapiura. There was a good correlation (R^2=0.64) between legume shoot dry weights and age of palms across legume species (Figure 4.7) dry weight decreased steadily with oil palm age after 5 years or more of growth, after which the shoot dry weight dropped to around 150 g/m^2 and remained steady during the mature years (>6 years old). Litter dry weights of the legume cover plants ranged from 100-804 g/m^2 and followed a similar pattern to the shoot dry weights (Figure 4.7) the weights were higher at 2-5 years of age and decreased steadily thereafter after this time and maintained a weight of around 100 g/m^2. There are high correlations as well with litter dry weights decreasing with oil palm age (R^2=0.6 and 0.7 for quadrat and plantation respectively). Litter dry weights ranged from 100 to 668 g/m^2 and were higher than shoot dry weight. *M. pruriens* was sampled under a 6 year old oil palm planting and had a mean shoot weight of 408 g/m^2 and litter dry weight of 668 g/m^2. All the measures of the shoot and litter dry weights of all the legume species are within two standard deviations from the mean (Table 4.3).

Table 4.3: Dry weight of the standing shoot and litter biomass in one square meter quadrat

Species Location	Age (years)	Shoot DM (quadrat g/m^2)	s.d.	Legume litter DM (quadrat g/m^2)	s.d.
C. caeruleum					
Mosa	4	363	200	551	173
Mosa	13	202	83	153	76
Mosa	18	144	56	184	110
Kapiura	3	421	174	489	244
Kapiura	5	176	26	159	75
Kapiura	7	150	80	100	108
Kapiura	25	161	40	119	35
Numundo	8				
Numundo	9		Data not collected	Data not collected	
Numundo	16				
P. phaseoloides					
Mosa	2	443	86	804	176
Mosa	4	362	98	694	205
Kapiura	3	371	83	589	267
Kapiura	5	267	64	467	229
Numundo	4		Data not collected	Data not collected	
M. pruriens					
Mosa	6	408	281	668	357
	mean	289		415	
	s.d.	117		257	
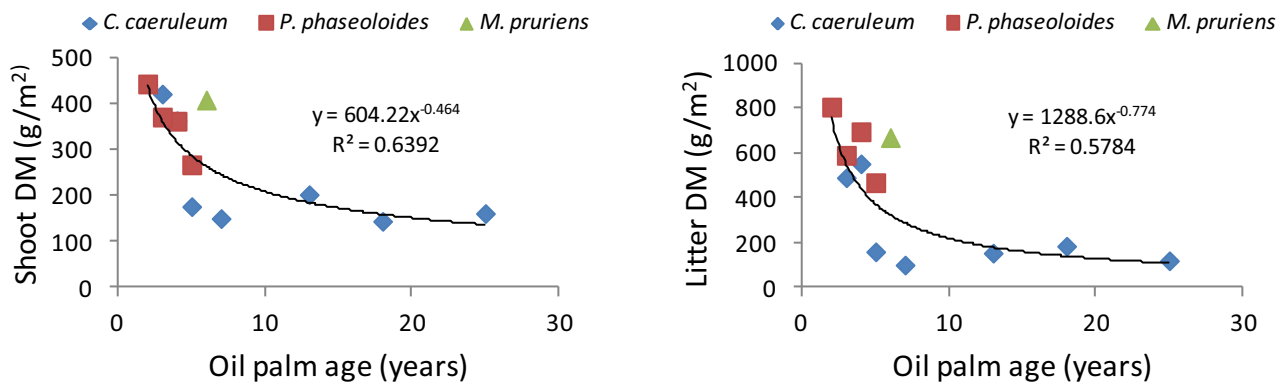
Figure 4.7: Dry weight of standing shoot and litter biomass (per m² quadrat) measured in oil palm plantations of different ages. Lines of best fit (power curves) were iftted to the data using Microsoft Excel

4.3.2 Shoot and litter N concentration (%)

The shoot N concentration ranged from 2.2 (*C. caeruleum*, Kapiura 25 year old) to 2.9% (*M. pruriens*, Mosa 6 year old) (Table 4.4). The litter N concentrations ranged from 1.6 to 3.2% with the higher values found in the 2-6 year old plantations and the lowest values in *C. caeruleum* in the mature plantations (>6 years old). There were poor correlations between N concentration in both shoot and litter with increasing age of palms. All of the values of the shoot and litter N concentrations are within two standard deviations from the mean.

4.3.3 Shoot and litter total N (g/m²)

Shoot N of the three legumes ranged from 3.6 (C. caeruleum, Kapiura 25 year old) to 12.1 (P. phaseoloides, Mosa 2 year old) g/m2 (Table 4.4). Legumes in the 2-6 year old plantations have higher shoot total N than in plantations more than 6 years old. Only C. caeruleum in a 5 year old planting had a lower shoot total N (4.5 g/m2). Litter total N within the legume quadrats ranged from 1.8 (C. caeruleum, Kapiura 7 year old) to 21.8 g/m2 (M. pruriens, Mosa 6 year old). The legumes also had higher litter total N in the 2-6 year old plantations following shoot total N: C. caeruleum in the Kapiura 5 year old planting had a lower litter total N (3.4 g/m2) similar to values obtained in plantations more than 6 years of age. There was good correlation

Figure 4.8) between shoot N with palm age (R^2=0.64) and litter N with palm age (R^2=0.57).

Table 4.4: Standing shoot and litter N concentrations (%) and total N (g/m^2) in legumes under different oil palm ages

Location	Age (years)	Shoot N (%)	s.d.	Shoot N (g/m^2)	s.d.	Legume litter N (%)	s.d.	Legume litter N (g/m^2)	s.d.
C. caeruleum									
Kapiura	3	2.4	0.2	10.1	3.9	2.2	0.2	10.7	5.4
Mosa	4	2.4	0.3	8.4	4.5	2.1	0.3	11.4	2.8
Kapiura	5	2.6	0.2	4.5	0.5	2.1	0.3	3.4	1.6
Kapiura	7	2.6	0.2	3.9	2.2	1.7	0.3	1.8	2.1
Numundo	8								
Numundo	9			Data not collected					
Mosa	13	2.6	0.4	5.2	2.0	1.9	0.2	2.9	1.5
Numundo	16								
Mosa	18	2.5	0.2	3.6	1.5	1.8	0.4	3.2	2.0
Kapiura	25	2.2	0.3	3.6	1.0	1.6	0.2	2.0	0.7
P. phaseoloides									
Mosa	2	2.7	0.3	12.1	2.6	2.2	0.1	17.9	4.5
Kapiura	3	2.3	0.2	8.6	1.8	2.3	0.3	13.4	6.2
Mosa	4	2.6	0.4	9.3	3.0	2.3	0.2	15.7	5.0
Numundo	4			Data not collected					
Kapiura	5	2.6	0.2	6.9	1.9	2.3	0.1	10.6	5.1
M. pruriens									
Mosa	6	2.9	0.3	11.9	8.1	3.2	0.5	21.8	13.6
mean		2.5		7.3		2.1		9.6	
s.d.		0.2		3.2		0.4		6.9	
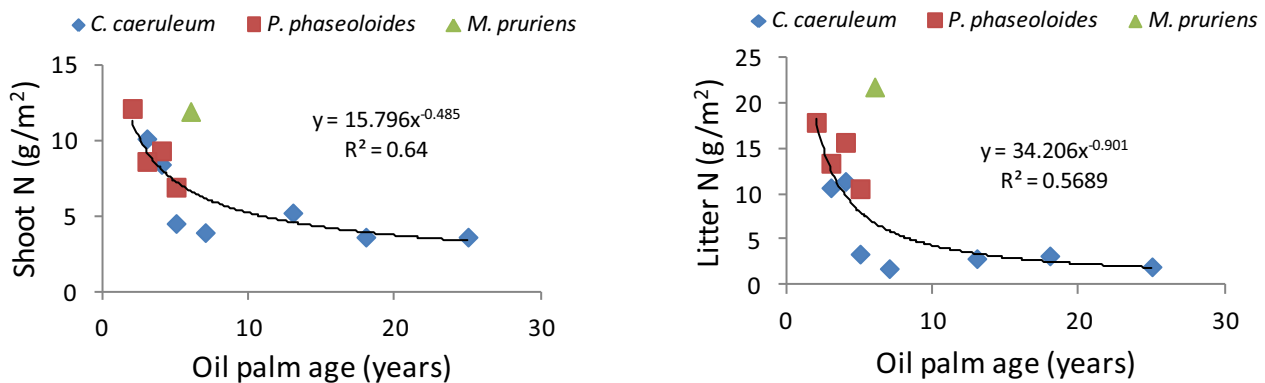
Figure 4.8: Correlation between legume shoot and litter total N (g/m²) in C. caeruleum, P. phaseoloides and M. pruriens with palm age. Lines of best fit (power curves) were fitted to the data using Microsoft Excel

4.3.4   Stem RU-N (%)and %Ndfa

C. caeruleum generally had higher stem RU-N than *P. phaseoloides* (Table 4.5), ranging from 17 to 43 and averaging 28.3%, while *P. phaseoloides* had a range of 13.2 to 37.9, averaging 18%. The stem RU-N for the only *M. pruriens* sampled was 28%. The glasshouse calibrations for *P. phaseoloides* and *C. caeruleum* were very similar, and a homogeneity of slopes analysis showed that they were not significantly different from each other (P>0.05 by analysis of variance), and thus they have been combined into a single calibration curve for field use for both species (Figure 4.9). The *M. pruriens* glasshouse calibration was quite different (see Chapter 2), reaching only 12% RU-N when the legume was totally dependent on N_2 fixation. This was less than half of the value recorded for the *M. pruriens* sampled in the field. As the glasshouse *M. pruriens* RU-N calibration curve was not able to be reconciled with the field observation of *M. pruriens* RU-N, the calibration in Figure 4.9 is used for all three species.

The dependence of *C. caeruleum* on N_2 fixation (%Ndfa) averaged 48.7% (range 28.4 - 74.8%) which was higher than that of *P. phaseoloides* which has a mean of 32% (range 18 – 65.7%) across the sites (Table 4.5). The measure of %Ndfa in *M. pruriens* in the single location sampled is 48.6. There were poor correlations (Figure 4.10) between stem RU-N and the age of palm (R^2=0.2) and between %Ndfa and age of palm (R^2=0.2) All the

measured values however, lie within two standard deviations of the mean, indicating similarity amongst the different species in their abilities to fix N_2.
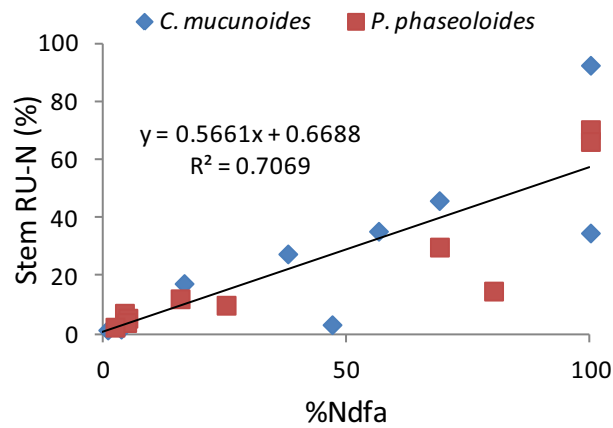
Figure 4.9: The combined ureide calibration equation for both C. mucunoides and P. phaseoloides obtained from the glasshouse experiment and applied in the field survey for all three species.
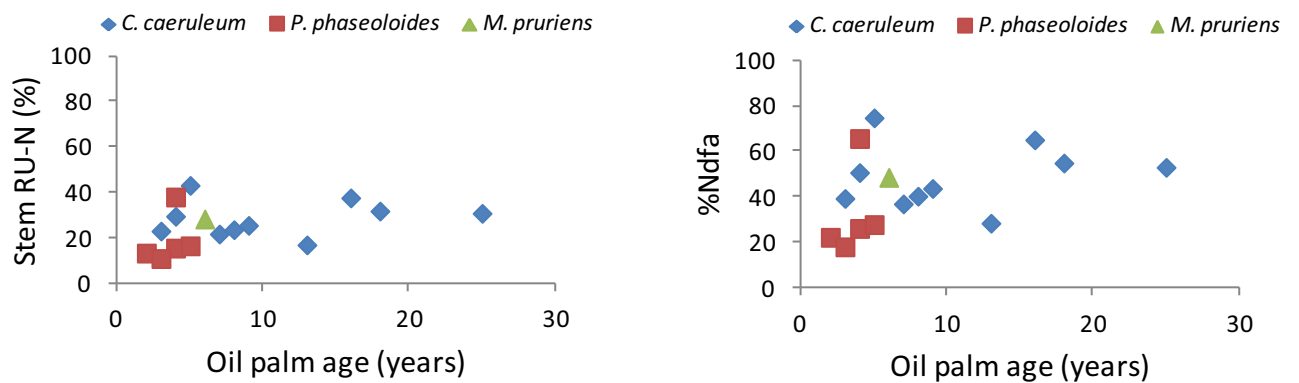
Figure 4.10: Relationship between age of palm and the stem RU-N (%) and %Ndfa values of the legumes under oil palm

Table 4.5: Stem RU-N (%), %Ndfa and fixed N measured in grams per square meter in standing shoot and litter biomasses of legumes under different oil palm ages

Location	Age (years)	Stem RU-N (%)	s.d.	%Ndfa	s.d.	Fixed N in shoots (g/m²)	s.d.	Fixed N in litter (g/m²)	s.d.	Fixed N in shoot+litter (g/m²)	s.d.
C. caeruleum											
Kapiura	3	22.9	7.1	39.3	12.6	4.4	3.2	3.2	2.3	7.6	5.0
Mosa	4	29.4	9.2	50.7	16.2	4.2	0.5	6.0	2.8	10.2	2.6
Kapiura	5	43.0	33.7	74.8	59.5	3.4	2.8	2.2	1.6	5.6	4.3
Kapiura	7	21.6	8.2	36.9	14.5	1.6	0.5	1.0	1.4	2.6	1.7
Numundo	8	23.5	3.6	40.3	6.4			Data not collected			
Numundo	9	25.4	11.9	43.7	21.0						
Mosa	13	16.8	10.9	28.4	19.3	1.5	0.9	0.9	0.7	2.4	1.5
Numundo	16	37.5	14.6	65.1	25.8						
Mosa	18	31.7	15.7	54.9	27.6	1.6	0.6	1.4	1.0	3.1	1.4
Kapiura	25	30.7	15.7	53.0	27.8	2.0	1.3	0.9	0.5	3.0	1.7
P. phaseoloides											
Mosa	2	13.2	4.0	22.2	7.1	3.1	1.0	4.0	0.9	7.1	1.8
Kapiura	3	10.8	4.0	18.0	7.0	1.6	0.6	3.0	1.8	4.6	2.2
Mosa	4	15.4	10.8	26.1	19.0	2.4	1.4	4.7	3.5	7.0	4.4
Numundo	4	37.9	12.4	65.7	21.9			Data not collected			
Kapiura	5	16.4	8.8	27.8	15.5	2.0	1.6	3.5	3.3	5.5	4.8
M. pruriens											
Mosa	6	28.2	9.6	48.6	16.9	3.8	0.6	5.9	2.8	9.7	3.4
	mean	25.3		43.5		2.6		3.1		5.7	
	s.d.	9.5		16.7		1.1		1.8		2.7	

4.3.5 Fixed nitrogen in shoot and litter (g/m²)

The amount of N_2 fixed in the shoot biomass was calculated as the product of %Ndfa and the total N (Table 4.5) estimated per square meter of legume, then scaled up to plantation using the legume ground cover estimates. Nitrogen fixation in standing shoot biomass ranged from 1.5 (*C. caeruleum*, Mosa 13 year old plantation) to 4.4 (*C. caeruleum*, Kapiura 3 year old) g/m², averaging 2.7 for *C. caeruleum*, 2.3 for *P. phaseoloides* and 3.8 for *M. pruriens*, but *M. pruriens* was sampled in only one location. It is not known what time period this might represent, due to turnover of shoots for these perennial species. However, if one were to assume that the %Ndfa of the standing shoot also applied to the accumulated legume litter, an amount could be added for the litter layer present. Thus an additional 0.9 to 6.0 g/m² of fixed N might have been fixed in the litter, giving a maxima of 2.4 (*C. caeruleum*, Mosa 13 year old) to 10.2 (*C. caeruleum*, Kapiura 3 year old) g/m² for shoot+litter fixed N (Table 4.5).

Table 4.6: Nodule assessment carried out for three legume cover plants under oil palm in Kimbe, WNBP in Papua New Guinea

Legume species			Nodulation	
	Location	Palm age	Score	Rating
C. caeruleum	Kapiura	3	5	excellent
C. caeruleum	Mosa	4	4	excellent
C. caeruleum	Kapiura	5	2	fair
C. caeruleum	Kapiura	7	2	fair
C. caeruleum	Mosa	13	4	excellent
C. caeruleum	Mosa	18	1	poor
C. caeruleum	Kapiura	25	3	good
M. pruriens	Mosa	6	2	fair
P. phaseoloides	Mosa	2	3	good
P. phaseoloides	Kapiura	3	5	excellent
P. phaseoloides	Mosa	4	3	good
P. phaseoloides	Kapiura	5	4	excellent

4.3.6 Nodule scoring

Nodule assessments for the legumes under oil palm in the different locations in Kimbe are given in Table 4.6. *Calopogonium caeruleum* had a score of 5/5 (excellent nodulation) in 3, 4 and 13 year old plantations while*P. phaseoloides* had excellent nodulation in 3 and 5 year old plantations in Kapiura. Nodulation was fair and good for *C. caeruleum* in 5, 7 and 25 year old Kapiura plantation. Nodulation in *C. caeruleum* in the Mosa old planting was poor (score: 1/5). *Pueraria phaseoloides* had good nodulation in 2 and 4 year old Mosa plantations while *M. pruriens* had fair to poor nodulation (2/5) in the 6 year old Mosa plantation. Generally nodulation was poorer in older plantations and better under the new plantations.

Table 4.7: Soil analyses data obtained from the sampling locations in the New Britain Palm Oil plantations in Kimbe, West New Britain Province

Species	Location	Year	Age (years)	Soil analysis (mg/kg)		
				Soil		
				pH	Nitrate	NH4+
C. caeruleum	Mosa	2008	4	5.1	7.3	192
C. caeruleum	Mosa	1999	13	5	17.1	95
C. caeruleum	Mosa	1994	18	4.8	3.5	131
C. caeruleum	Kapiura	2009	3	4.3	11.7	134
C. caeruleum	Kapiura	2007	5	4.9	5	292
C. caeruleum	Kapiura	2005	7	4.6	5.2	151
C. caeruleum	Kapiura	1987	25	4.7	2.2	220
C. caeruleum	Numundo	2003	8			
C. caeruleum	Numundo	2004	9	data not collected		
C. caeruleum	Numundo	1996	16			
P. phaseoloides	Mosa	2010	2	4.4	14.6	166
P. phaseoloides	Mosa	2008	4	5	7.3	197
P. phaseoloides	Kapiura	2009	3	4.4	24.5	245
P. phaseoloides	Kapiura	2007	5	5.2	6.6	252
P. phaseoloides	Numundo	1998	4	no data		
M. pruriens	Mosa	2006	6	5.6	7.1	233

4.3.7 Soil analysis

Soils were taken from all the areas surveyed for N_2 fixation by the legume cover plants and analysed for pH, nitrate+nitrite and ammonium (Table 4.7). The soils from all the areas were acidic in nature with a pH range of 4.3-5.6. Soils from the youngest plantation (2-3 year old) were slightly more acidic than the others, apart from that there were no other obvious trends in the acidity of the soils, either due to different legume species nor survey sites.

(a)   (b)
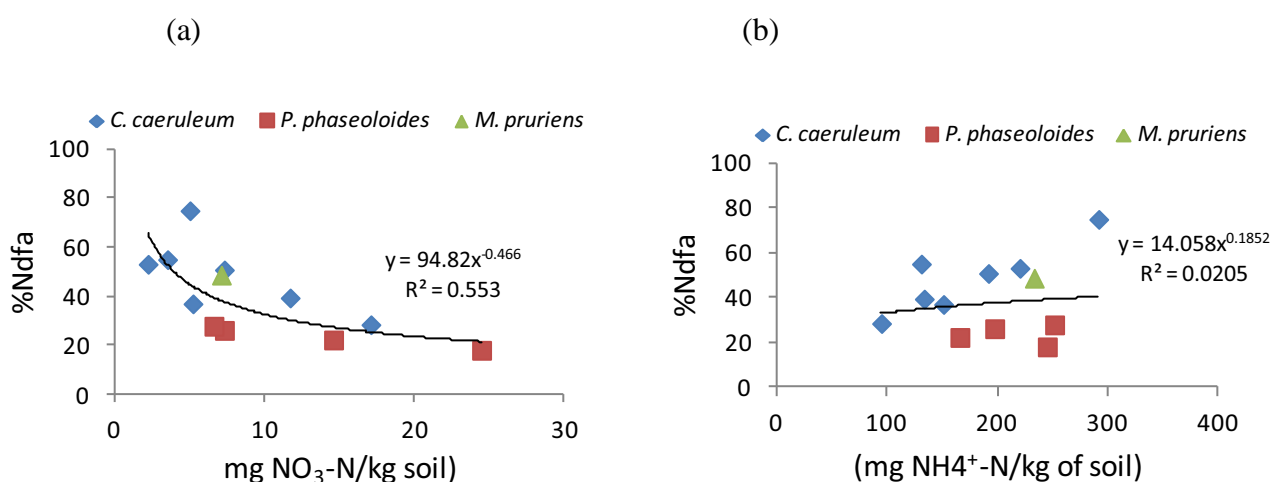
Figure 4.11: Relationship between %Ndfa of species under the oil palm plantations and (a) (soil nitrate-N or (b) soil ammonium-N concentration. A power curve is fitted to the data across all three species. Lines of best fit (power curves) were fitted to the data using Microsoft Excel

All the soils were exceptionally high (95-292 mg NH_4^+-N/kg) in ammonium, but much lower nitrate+nitrite (range: 2.2-24.5). The highest nitrate (24.5 mg NO_3^--N/kg) was found in the 3 year old Kapiura plantation where *P. phaseoloides* was sampled and the lowest (2.2 mg NO_3^--N /kg) in the 25 year old Kapiura plantation where *C. caeruleum* was sampled. There was a strong relationship (R^2=0.55) between soil nitrate concentration and dependence on N_2 fixation (Figure 4.11).

Table 4.8: Plantation standing shoot and litter dry weights (kg/ha) of legumes under different oil palm ages

Species Location	Age (years)	Plantation legume cover (%)	s.d.	Plantation legume shoot DM (kg/ha)	s.d.	Plantation legume litter DM (kg/ha)	s.d.
C. caeruleum							
Mosa	4	19.7	13.3	854	919	1132	842
Mosa	13	4.7	5.5	99	112	73	81
Mosa	18	10.2	15.5	167	301	308	539
Kapiura	3	8.7	2.2	373	192	406	224
Kapiura	5	18.8	3.0	332	75	293	131
Kapiura	7	7.4	5.7	94	63	54	45
Kapiura	25	0.6	1.0	9	14	7	12
Numundo	8						
Numundo	9		2.9	Data not collected	Data not collected		
		1.7					
Numundo	16	1.4	1.7				
P. phaseoloides							
Mosa	2	44.0	8.1	1983	668	3610	1352
Mosa	4	2.4	3.1	73	86	160	215
Kapiura	3	8.1	2.7	295	107	492	316
Kapiura	5	9.5	4.6	243	111	367	90
Numundo	4		2.4	Data not collected	Data not collected		
		3.9					
M. pruriens							
Mosa	6	5.1	5.1	276	447	394	548
	mean	9.7		400		608	
	s.d.	11.1		545		990	

4.3.8 Plantation legume cover (%)

Legume cover ranged from 0.6 (*C. caeruleum*, Kapiura 25 year old) to 44% (*P. phaseoloides*, Mosa 2 year old), declining with age of plantation (Table 4.8). There were insufficient samples in the survey to be able to ascertain if there were trends in species presence with age of oil palm, *M. pruriens* was only encountered in one plantation. From the data collected, more legume cover was recorded in the 1-6 year old plantations and only

C. caeruleum was evident under the plantations more than 6 years old. Generally the percentage of legume as ground cover decreased as oil palm matures (R^2=0.4) (Figure 4.12).
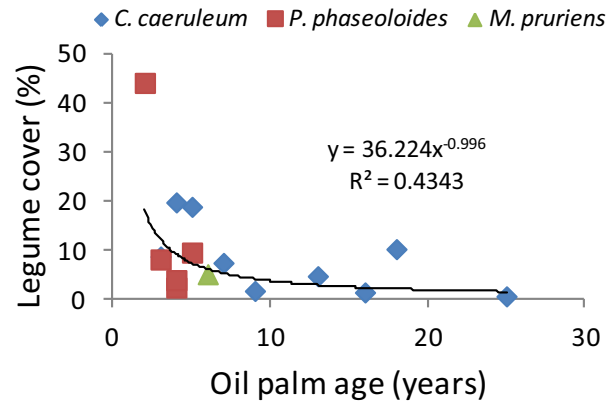
Figure 4.12: Correlation between legume cover and oil palm age. A power curve is fitted to the data across all three species. Lines of best fit (power curves) were fitted to the data using Microsoft Excel

4.3.9 Shoot and litter dry matter (kg/ha)

When converted to a plantation basis trends were similar to those of the quadrat data. Legume shoot biomass ranged from 9 to 1983 kg/ha of plantation (Table 4.8). The lowest being *C. caeruleum* in the 25 year old Kapiura plantation while *P. phaseoloides* had the highest shoot DM in the 2 year old Mosa plantation. There was a good correlation (R^2=0.6) between legume shoot biomass and age of palms across legume species (Table 4.8). Legume litter dry weights showed the same trends, ranging from 7 to 3610 kg/ha (Figure 4.13), being higher at 2-6 years of age and decreased steadily after this time. *M. pruriens* had a mean shoot weight of 276 kg/ha and litter dry weight of 394 kg/ha.

4.3.10 Shoot and litter total N (kg/ha)

The total N in the standing shoot biomass of the legumes ranged from 0.2 (*C. caeruleum*, Kapiura 25 year old) to 54.2 kg/ha (*P. phaseoloides*, Mosa 2 year old) (Table 4.9) and followed the same trends as shoot biomass in regard to oil palm age. The total N measured in the legume litter were within the range of 0.1 (*C. caeruleum*, Kapiura 25 year old) to 80.8 kg N/ha (*P. phaseoloides*, Mosa 2 year old). Legume shoot and litter in the 2-6 year old plantations contain more than 6.0 kg of N/ha except for *P. phaseoloides* in the 4 year old Mosa plantation (<4 kg N/ha). There was high correlation (R^2=0.6) between litter N and oil palm age; litter N decreases with oil palm age (Figure 4.13).
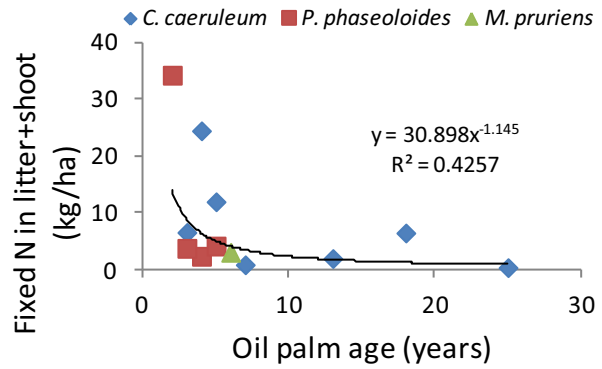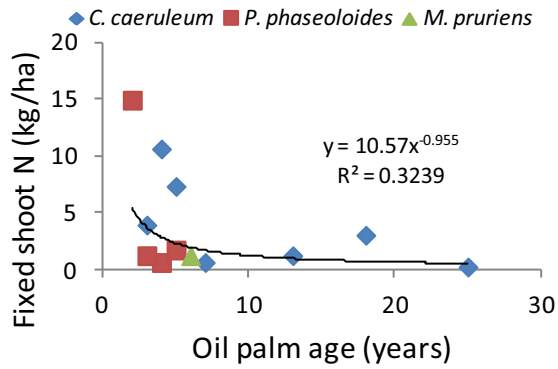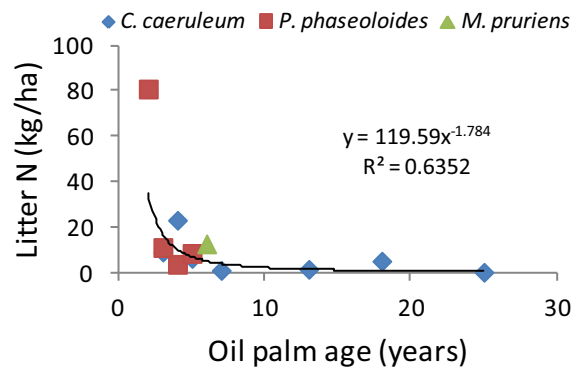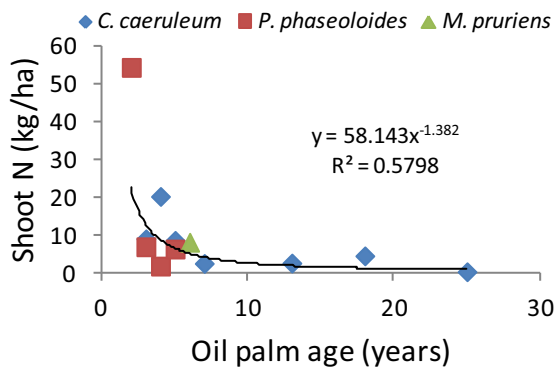
Figure 4.13: Dry matter, total N and total fixed N in the standing shoot and litter biomass of legumes in the plantation presented in kilograms per hectare and power regressed with oil palm age. Lines of best fit (power curves) were fitted to the data using Microsoft Excel

Table 4.9: Fixed and total N measured in kilograms per hectare in standing shoot and litter biomasses of legumes under different oil palm ages

Location	Age (years)	Plantation legume shoot N (kg/ha)	s.d.	Plantation legume litter N (kg/ha)	s.d.	Fixed N in legume shoots in plantation (kg/ha)	s.d.	Fixed N in legume litter in plantation (kg/ha)	s.d.	Fixed N in legume shoot + litter (kg/ha)*	s.d.
C. caeruleum											
Kapiura	3	8.9	4.4	9	5.1	3.9	3.4	2.6	2.3	6.5	5.2
Mosa	4	20.1	21.8	23	16.8	10.6	4.2	13.8	6.1	24.4	6.3
Kapiura	5	8.5	1.7	6.2	2.7	7.3	6.2	4.6	3.4	11.9	9.5
Kapiura	7	2.4	1.6	0.9	0.6	0.6	0.4	0.2	0.1	0.8	0.5
Mosa	13	2.5	2.7	1.4	1.6	1.2	1.4	0.7	1	1.9	2.5
Mosa	18	4.4	8.1	5	9.2	3	4.8	3.4	5.4	6.4	10.2
Kapiura	25	0.2	0.3	0.1	0.2	0.2	0.3	0.1	0.1	0.3	0.4
P. phaseoloides											
Mosa	2	54.2	18.7	80.8	33.1	14.9	4.8	19.3	4.9	34.2	8.4
Kapiura	3	6.8	2.2	11	7	1.2	0.6	2.5	1.9	3.7	2.4
Mosa	4	1.7	1.9	3.6	4.7	0.6	1	1.7	3.3	2.3	4.3
Kapiura	5	6.2	2.6	8.4	2.3	1.7	0.7	2.4	1.6	4.1	2.1
M. pruriens											
Mosa	6	8	12.9	12.6	19.2	1.2	1.6	1.7	2.5	3	4
	mean	10.3		13.5		3.9		4.4		8.3	
	s.d.	14.8		22.1		4.7		5.9		10.5	

4.3.11 Fixed N in shoot and litter (kg/ha)

The amount of fixed legume N for standing shoot and litter in the plantation was calculated as the product of %Ndfa, total N and percentage of legume cover (Table 4.9). Nitrogen fixation in standing shoot biomass ranged from 0.2 (*C. caeruleum*, Kapiura 25 years old) to 14.9 (*P. phaseoloides*, Mosa 2 years old) kg/ha, averaging 3.8 for *C. caeruleum*, 4.6 for *P. phaseoloides* and 1.7 for *M. pruriens*. Due to the turnover of shoots, the time period this represents is not known. A value of fixed N for plantation litter was estimated using the same %Ndfa as the standing shoot biomass giving an additional 0.1 to 19.3 kg/ha of fixed N which gave a maxima of 0.3 (*C. caeruleum*, Kapiura 25 years old) to 34.2 (*P. phaseoloides*, 2 years old) kg/ha for shoot+litter fixed N. There was a weak correlation (R^2=0.3) between the amount of fixed N in the legume shoot with palm age, and between shoot+litter N fixation and palm age (R^2=0.4) (Figure 4.13).

4.4   Discussion

4.4.1   Legume cover DM under oil palm in PNG

Overall, legume growth under oil palms in PNG was good, with legume covers having higher standing biomass in the early years of oil palm establishment and lesser in the later years, presumably largely due to the shading effect of the expanding oil palm canopy. The tropical climate in PNG, with high rainfall resulting in low vapour pressure deficit, long sunshine hours and a 23°C daily mean temperature (Banabas 2007), is a natural habitat for these tropical legumes (Giller 2001b) and ideal for maximising growth. The fertilizer applications (Table 4.1) would have ensured adequate nutrient supply and contributed to optimal growth. Indeed, the highest shoot biomass, measured for *P. phaseoloides* of 1983 kg/ha (at 44% cover) was close to the 1950 kg/ha recorded for the same species grown as monoculture in the wet season in the savannah of Colombia (Cadisch *et al.* 1989). Climatic and environmental conditions for PNG and the study site in Colombia are generally similar: annual rainfall patterns of >2000 mm, similar sunshine hours and temperatures and well drained soils. In Colombia, the legume received 70 kg fertiliser K/ha (but no P) and the legume was inoculated with a *Bradyrhizobium* strain before being sown. Fertiliser K application in the PNG oil palm plantations was nearly twice as much as that used in the study in Colombia (134 kg K/ha calculated from Table 4.1 with oil palm planting density of 132 palms/ha), which may account for the slightly higher shoot biomass. Whilst *P. phaseoloides* was a monoculture crop in the Colombian study, the productivity can be

114

compared with that in the current study since *P. phaseoloides* was largely growing alone in the young plantations sampled. It could be said from these similarities that PNG field conditions had sufficient nutrient supply, optimum climatic conditions for legume growth and good rhizobial populations as well. In fact, *P. phaseoloides* shoot biomass in PNG was calculated at a mean of 14% ground cover to be just over half a tonne per hectare whilst presumably Cadisch, Sylvester-Bradley & Nosberger (1999) would have estimated for a 100% legume ground cover. This suggests *P. phaseoloides* in the current study was much more productive than in the study in Colombia, at around 3.5 tonnes if the mean values extrapolated to 100% cover. Shoot biomass in Colombia did increase to 3842 kg/ha when 80 kg K/ha was applied as fertiliser indicating an influence of limited K supply on growth. Other work (Reynolds 1982) examining the effect of N fertiliser applications on productivity of grass-legume mixtures reported higher shoot dry matter productivity than in this present study for *P. phaseoloides* (4133 kg/ha) and *C. mucunoides* (5673 kg/ha) grown in mixtures with two different pasture grass species. This standing shoot DM is higher than the maximum legume shoot DM in the current study, but again if the legume cover estimation is considered, the legume growth in PNG is similar to that recorded by Reynolds (1982). There are a number of other studies that measured shoot biomass production of *P. phaseoloides* grown in mixtures and intercropped situations (Gil *et al.* 1997; Tian *et al.* 1999; Viera-Vargas *et al.* 1995; Zaharah *et al.* 1986). The highest *P. phaseoloides* shoot biomass recorded (756 g/m^2) was after 1 year and 8 months of growth by Tian et al (1995). These plots had regular weeding but no fertilizer application. The highest *P. phaseoloides* biomass in the current study (443 g/m^2) is almost half of that by Tian et al (1995).

Total turnover of legume shoot biomass is unknown in the current study, as is the growth time period that the sampled standing biomass represents. The above-mentioned studies, with similar legume productivity to the current study, generally reported 4-6 months of biomass production. According to Vesterager et al (1995), *P. phaseoloides* litter starts to accumulate about 6 months after establishment, which suggests that the standing biomass measured for *P. phaseoloides* in PNG may cover 6 months of growth or less, if *P. phaseoloides* standing biomass reaches maximum growth at 6 months. When extrapolating the legume standing biomass (shoot and litter) to plantation level (kg/ha) the estimations reflected percentage of legume cover which was patchy because of the existence of non-cover areas such as harvest paths, weeded circle and frond piles. This patchy cover, partially imposed by management, is a limit to the potential N_2 fixation input of the system.

The productivity of legumes generally decreased with the age of plantation, both in terms of biomass per square metre and percentage legume cover. Shading could have affected the growth and occurrence of *P. phaseoloides*, *C. mucunoides* and *M. pruriens*, since only *C. caeruleum* is shade tolerant (Giller 2001b) and thrived under the older plantations where the oil palm canopy created shaded understorey (**Error! Reference source not found.**). Oil alm plantations generally grow larger canopies with increased age which induces more shade under the palms. The effect of shading has been studied by many. Sprent and Silvester (1973) demonstrated the effect of shading by *Pinus radiata* on decreasing N_2 fixation in *Lupinus arboreus*; Broughton (1977) also reported that competition from both the shade and roots of rubber plantations caused *C. mucunoides*, *C. pubescens* and *P. phaseoloides* to virtually die out about the 6th year after planting. Wilson and Wong (1982) demonstrated that reduced sunlight (by 60% and 40%) decreased dry matter in a mixed sward of the legume Siratro and grass green panic by 10-12%; legume growth alone was reduced to 33-38%. Similar work by Lin et al (1999) with different levels of shade (full sun, 50 and 80% induced shade) in a greenhouse on 30 different forage grass and legume species showed both positive and negative effects of shade depending on the plant species.

4.4.2 Legume cover N content under oil palm in PNG

Total N content in plants is a function of the dry matter and N concentration. In this study the N concentration (%) in the standing shoot and litter biomasses of the covers did not decline with age with all legume species sampled maintaining a fairly constant tissue N concentration. Legumes may exhibit subtle fluctuations in N concentration at the time of N fertilizer application or peak N_2 fixation; however, generally legumes tend to maintain their tissue N concentration relatively constant through N_2 fixation (Duthion and Pigeaire 1993). The mean N concentration of the legumes (2.4%) is within the sufficient N concentration range for herbaceous broadleaf ground covers (2.0 to 3.9%) according to Barker and Bryson (2007) and nearly similar to the mean shoot N (2.69) reported for Australian field grown legume crops and pastures (Unkovich et al 2010). Since nodulation tended to be good for legumes under younger plantations it could be postulated that the legumes primarily depended on N_2 fixation at this time to maintain this healthy tissue N whereas in older plantations, where nodulation of cover legumes was observed to be poor, N derived from fixation may have been supplemented with uptake of N from soil. However, whilst there was a negative correlation between soil nitrate and %Ndfa there was no consistent pattern of higher nitrate in older plantations.

Plate 8:*Calopogonium caeruleum* in (a) a four year old and (b) an 18 year old plantation

(a)

(b)
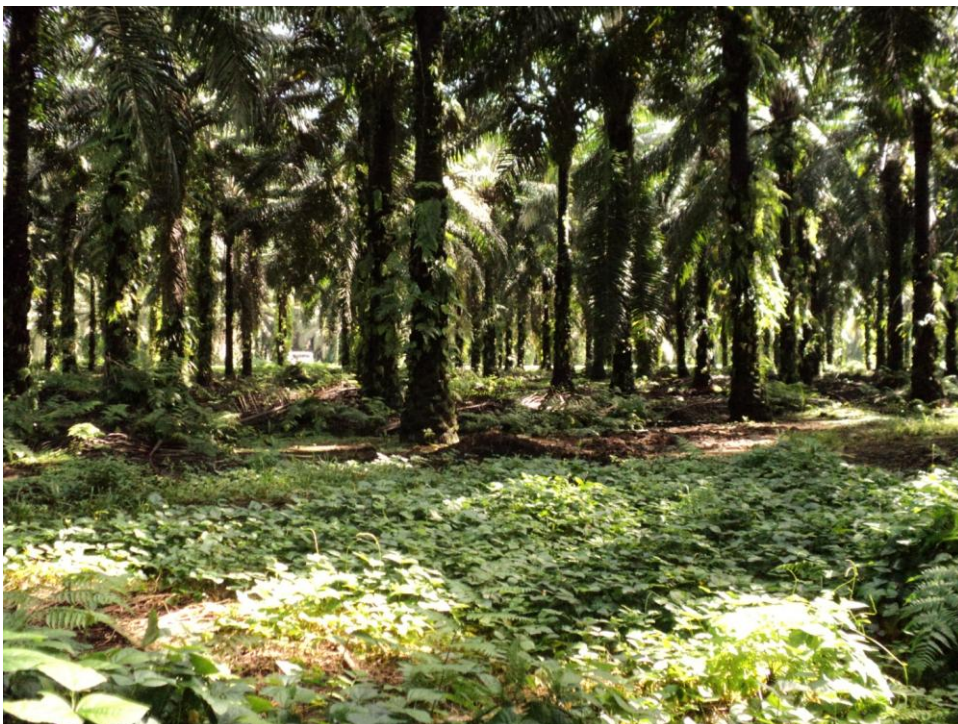
During the first 6 years of growth (0 to 6 year old plantations), the legumes accumulated a mean of 14.3 kg N/ha (54.2 kg N/ha maximum). If the total N contained in litter is added, the amount is tripled (mean: 33.6 and maximum: 135 kg N/ha). These amounts are underestimates since the number of replicates was small and the total N in roots was not determined. The estimates of legume total N are still substantial compared to the current mean mineral N application of 124 kg N/ha at 132 palms/ha planting density (for 0-6 year old palms) (see Table 4.1). Further work needs to be carried out to establish the amount of N accumulated in legume biomass within a known time frame. With this information as well-supported evidence, recommendations on replacing the mineral fertilizer N with input f fixed N via legumes, possibly during the first 4 to 6 years of oil palm growth could be made for the PNG oil palm industry. A related experiment in a 2 years old oil palm plantation by Zahara $et\ al$ (1986) showed that 37.8 kg N/ha was accumulated in 3 months of $P.\ phaseoloides$ regrowth from a standardised cut, which promises that there is higher N accumulation potential in legumes under PNG oil palm system. When related to other measures of legume total N in other studies, the current estimations are within accepted ranges. The shoot N range within a 1 m^2 quadrat (3.6 to 12.1 g/m^2) relates well to the values obtained for $C.\ mucunoides$, $M.\ pruriens$ and $P.\ phaseoloides$ in other researches (Cadisch $et\ al.$ 1989; Fosu 2003; Giller 2001a; Mendham $et\ al.$ 2004; Viera-Vargas $et\ al.$ 1995). The higher total N values (range 15 – 27 g/m^2) recorded for $P.\ phaseoloides$ in other findings (Franke $et\ al.$ 2008; Sylvester-Bradley 1984; Tian $et\ al.$ 1999) during shorter study periods (as short as 3 months) suggest that the N accumulation potential of $P.\ phaseoloides$ and the legumes used under the PNG oil palm system could be higher than currently estimated.

4.4.3   Legume cover -proportional dependence on N_2 fixation

The calibration of the RUN technique to estimate N_2 fixation by the legume cover plants was successfully applied for the first time for these species and also for the first time in PNG, although there is some small element of caution with respect to the calibration of the ureide assay in less than optimal growth conditions in the glasshouse. This presents a valuable tool for assessing N_2 fixation particularly since a pilot study assessing delta ^{15}N values for plants in these systems (McNeill et al unpub. data) concluded that the natural abundance technique could not be used on West New Britain due to low delta ^{15}N values of reference plants (Unkovich $et\ al.$ 2008). There was one other work published that measured

ureides levels in *P. phaseoloides* and *C. mucunoides* but this did not include ^{15}N calibration. Norhayati et al (1988) measured stem RU-N in a range of legume species and showed that *C. caeruleum* and *P. phaseoloides* had wider ranges of stem RU-N values (10.6 to 69.2 and 19.8 to 75.8 respectively) than in the current study (Table 4.5) and related those variations to inoculation and N fertilizer treatments. The field stem RU-N found in the current study for *P. phaseoloides* (10.8 to 37.9%) and *C. mucunoides* (16.8 to 43.0%) could be higher as shown by Norhayati et al (1988) and the glasshouse values (>60%) reported in Chapter 2. Some limiting factors such as high soil nitrate and soil acidity (Table 4.7) may have reduced dependence on N_2 fixation for these species in the field. There may also be low populations of compatible rhizobia in some locations which resulted in low stem RU-Ns but no rhizobial studies have been carried out on these soils to verify this. Since the PNG soils are highly acidic rhizobial populations could have been affected.

On average 41.3 % (mean %Ndfa from the current survey) of the legume cover total N discussed in the previous section came from N_2 fixation, with ranges from 18-74%. This is important to note so that contributions from legumes could be distinctive from mineral fertilizer contribution and thus its use could be maximized. Dependence on N_2 fixation did not appear to decline with legume age although it was noted that nodulation tended to be better in covers in younger plantations. *Calopogonium caeruleum* was the main legume found under older plantations and maintained a constant dependence on N_2 fixation. Being a shade tolerant species *C. caeruleum* was established primarily for the purpose of weed suppression and N_2 fixation (Fairhurst and Hardter 2003) and its predominance under the older oil palm plantations was expected. The older plantations tend to have more missing palms due to deaths caused by pests and diseases which, as was observed during the field survey, allows for more sunlight under the oil palm canopy to better support legume growth and N_2 fixation. However, there were no measures of light intensity or N_2 fixation in areas of missing palms under oil palm carried out during this study, and it is suggested that measures like this could clarify the influence of shade and N contributed by *C. caeruleum*.

As with stem RU-N, it was expected that the %Ndfa might be higher in all the legume species sampled. Other studies (Fosu 2003; Giller 2001a; Sanginga 2003; Sanginga *et al.* 1996a; Sanginga *et al.* 1996b) have found *M. pruriens* to have higher ranges of %Ndfa (range 60-91%) compared to the *M. pruriens* mean in the current study (48.6%). *P. phaseoloides* was also recorded to have values as high as 87% (Cadisch *et al.* 1989) and *C.*

mucunoides 79% (Giller 2001a) in field surveys. Fosu (2003) measured N_2 fixation (using ^{15}N natural abundance) to be 81.3% in *M. pruriens* in Northern Ghana 84 days after sowing with P and K applied at 3.3 and 16.4 kg/ha respectively and no applied N. *M. pruriens* was found (using ^{15}N isotope dilution) to fix 86% of its total N 12 weeks after planting when inoculated and grown in the field with maize while having a lower %Ndfa (63-74%) when grown uninoculated, fertilized with N and grown alone. A blanket application of 30 kg/ha of P and K, respectively, were applied as well (Sanginga *et al.* 1996a). The higher %Ndfa obtained in Fosu (2003) and Sanginga et al (1996) was positively influenced by low soil nitrate and the combined effect of inoculation and competition by maize. Similar situation was observed in the current study where %Ndfa was affected by the higher soil nitrate (Figure 4.11). As stated above the soil nitrate affected N_2 fixation in the current study as well and soil acidity could be the other main limiting factor as well. Since there is evidence that soil nitrate is the main limiting factor on N_2 fixation by legumes under PNG oil palm plantations, the application of N fertilizers should be managed with consideration of this fact. Banabas (2010) also showed that large amounts of nitrate (96 and 20 kg/ha/year in Dami and Sangara respectively) are leached beyond the oil palm root system so a balance could be struck between fertilizer N application and maximising the N input from legumes. Measures such as discontinuity of fertilizer N application in the frond tips areas where legumes grow (Figure 4.4). in the plantations could be introduced so that legumes could fix N with minimal effect from fertilizer N. Fertilizer N could be applied instead in the frond pile areas away from the legume cover. Nitrogen fertilizers that are less acidic like ammonium nitrate and ammonium chloride could also be used instead of ammonium sulphate or di-ammonium phosphate in order to reduce acidification of the soils as well and allow optimum soil pH for rhizobial activities to occur, in turn encouraging N_2 fixation.

Considering the individual species in this survey, *C. caeruleum* had a higher range of %Ndfa (28.4 to 74.8) compared to *P. phaseoloides* (18.0 to 65.7%) although this could be confirmed further with more replicate samplings and statistical analysis. Further studies should be carried out to see what factors (i.e. soil pH, soil chemical analysis, soil rhizobial populations etc) might be favoured by *C. caeruleum* to encourage higher dependence on N fixation while they were not so for *P. phaseoloides*. According to Sylvester-Bradley et al (1991), *P. phaseoloides* is "promiscuous ineffective" which means that it can nodulate with a wide range of bacteria but many of them are ineffective at N_2 fixation; this may be a possible factor in PNG soils, and should be investigated. The high %Ndfa value (65.7%)

recorded for *P. phaseoloides* was also assumed to belong to *C. mucunoides* since these two species could not be distinguished, but other studies have shown that *P. phaseoloides* is capable of higher dependence on N_2 fixation.

4.4.4 Amount of N fixed by legume covers

Amounts of N fixed per hectare of plantation in standing shoot biomass decreased from 14.9 to 0.2 kg N/ha because dry matter productivity declined over time. The amount of N fixed per hectare of plantation was also a function of the percentage of legume cover, so even if the dependence on N_2 fixation for a legume species was high, scarcity of that species would result in low fixed N per hectare. The highest recorded legume cover was 44% with values as low as 2% . For future studies an increase in the number of sites and replicates sampled would be encouraged which might improve estimates of mean percentage legume cover and values for fixed N per hectare. Other studies have shown that these species: *C. caeruleum*, *P. phaseoloides*, *M. pruriens* and *C. mucunoides* are capable of fixing higher kg of N per hectare. Cadisch, Sylvester-Bradley & Nosberger (1999) showed that in 17 weeks, field grown *P. phaseoloides* in Colombia fixed a maximum of 115 kg N/ha when fertilized with P and K at 80 and 70 kg/ha respectively; *C. mucunoides* fixed 59.4 kg N/ha in 84 days at 17 and 33 kg/ha of P and K respectively and with no added nitrate (Fosu 2003) 3.6.4 and *M. pruriens* fixed 238 & 252 kg N/ha in 12 weeks in West Africa, when inoculated and not fertilized with nitrate respectively (Sanginga *et al.* 1996a). These figures were estimated with the percentage legume cover taken as 100%. The P and K nutrients are also applied in PNG so nutrients should be sufficient for the legumes under oil palm, but as mentioned before, soil nitrate levels reduced pNdfa and high soil acidity could also be a limiting factor too.

Increasing the amount of legume cover per hectare especially in the plantations <6 years old and establishing more shade tolerant legume species such as *C. pubescens* and *C. caeruleum* could be a way forward to increase the amount of N fixed per hectare in PNG oil palm plantations achieve this. Another advantage of using *C. caeruleum* is that the litter decomposes slower than *P. phaseoloides* (Giller and Wilson 1991) so it can contribute N to the oil palm system for a longer period. Seed supplies of *C. caeruleum* into the PNG oil palm plantations were reduced for the last 5-6 years due to short supplies but this could be resolved by sourcing the seeds locally.

The measures of biomass production, legume %N, legume total N, relative ureide-N, %Ndfa and fixed N do not include the turnover of shoot into litter, litter turnover or input of legume root N. Estimates of litter declined with increasing plantation age, nevertheless if the amounts of fixed N in litter are taken into account, the quantities of N fixed calculated in this survey increase by 33 - 283% and are 0.3 - 34.2 kg N/ha compared to 0.2 - 14.9 kg N/ha based on shoot only. The amount of fixed N in the litter could have accumulated over a longer time period, being higher than the shoot fixed N. However, these estimates still do not fully represent the total amounts of N fixed due to the high turnover rates of litter and biomass in these systems and the exclusion of legume root N which could increase the amounts considerably. For example, root N may constitute around 30% of total legume N according to (Unkovich *et al.* 2010b) and if this was accounted for then the estimates of N fixed by legume covers in PNG would range from 0.4 to 44.5 kg N/ha. Potentially this will still be a conservative estimate given that improvements discussed previously could increase.

The soil data showed that all the soils in the areas sampled for N_2 fixation were highly acidic (pH range: 4.3 to 5.6) and that decreasing %Ndfa was directly related to increasing nitrate in the soils. These soils would have sustained two to three oil palm plantings which means the soils have been receiving potentially acidifying fertilizers for 25 (lifespan of an oil palm planting) to 50 years. Some fertilizers that have been applied such as ammonium sulphate are mostly acidifying even if the uptake by palms is good (Nelson *et al.* 2010). Soil acidity and nitrate concentration have been discussed above in their effect on N_2 fixation by the legume cover plants in this current study. Acidity affects the exchange of the molecular signals between the legume and the micro-symbiont amongst other things (Hungria and Vargas 2000) and might restrict nodulation and N_2 fixation in PNG oil palm soils.

Ammonium-N (NH_4^+) in these soils appeared very high (95 to 292 mg/kg soil) but this was likely to be an artefact of sample processing rather than representative of real conditions in the field. Soils from the current study were irradiated at 25kGray on importation to Australia and due to delays in release of the treated soil samples from the place of irradiation it was nearly three months before the soils were analysed. Lensi et al (1991) reported a substantial increase in ammonium concentration in irradiated soils (at 25kGray)

as the incubation time increased from day 0 when the soils were irradiated to 55 days later (from 28 to 149 µg NH_4^+/g soil), especially when wet. The soils for this study had been oven dried but under humid atmospheric conditions in PNG may have absorbed moisture before being sent to Australia. The increase in NH_4^+ is partly a result of release of amino N from soil organisms killed during irradiation, and subsequently ammonium is not converted to nitrate due to the absence of activity from nitrifying bacteria. In addition it is also suggested that enzymatic activity can continue in the irradiated soils to some extent and cause deamination of nitrogenous organic compounds, which will further increase NH_4^+ concentration (Lensi et al 1991). Irradiation appears to largely have little effect on soil nitrate concentration (McNamara *et al.* 2003).

4.4.5 Conclusion

Legume cover dependence on N_2 fixation did not decrease with plantation age whilst amounts of N fixed did decline in the older plantations due to reduced legume cover and productivity. It is suggested that competition with oil palms for soil mineral N in the older plantations may have caused legume covers to remain reliant on N_2 fixation, although nodulation appeared better in younger legume covers. Efforts to increase N_2 fixation should primarily focus on increasing legume cover. This may be achieved by making sure that legume cover seeds are always sown during the establishment of new oil palm planting. A study of legume growth across a range of soil types that support oil palm systems in PNG, with and without fertilizer treatments, would establish knowledge on legume cover biomass production in PNG. Such an experiment could use N fertiliser rates to determine how soil N affects dependence on N_2 fixation by legume covers. Elsewhere *M. pruriens* has demonstrated an ability to fix higher amounts of N_2 than observed in the current study and could be further studied on PNG soils to assess its potential as a N_2 fixer for the oil palm system. In addition, a study of the rhizobial populations in the PNG oil palm soils could provide useful information on species present. *M. pruriens* did not have good nodulation so the study could also search for compatible rhizobial populations for *M. pruriens*. The calibration of the stem ureide technique means it can be confidently used by others for estimating biological N_2 fixation in *P. phaseoloides* and *C. mucunoides*. *M. pruriens*, on the other hand, requires a different approach to measure N_2 fixation, and other methods will have to be assessed.

5 General discussion

Nitrogen is a major nutrient that is needed by palms to be highly productive but loss from soil, to waterways and air could compromise environmental health. Legume cover plants that are grown under oil palm to suppress weeds and control erosion also fix atmospheric N_2 and ultimately, via residue decomposition, make it available for palms to take up. Before N in oil palm systems can be sustainably managed, it is necessary to quantify the input of N by biological N_2 fixation, the turnover of legume N to soil organic matter and the ultimate contribution to oil palm N nutrition In order to measure N fixation, a glasshouse experiment was set up in which the stem ureide proportions was calibrated against the ^{15}N isotope dilution technique. The calibrations were then applied in PNG oil palm plantations to measure N_2 fixation by legume cover plants *C. caeruleum*, *C. mucunoides*, *P. phaseoloides* and *M. pruriens*.

The calibration of the ureide technique was successful in the glasshouse study. The %Ndfa obtained by the ^{15}N isotope dilution was strongly correlated with stem RU-N, enabling calibration equations to be derived for the first time for these species. Since there were no significant differences between *P. phaseoloides* and *C. mucunoides* in their stem RU-N and %Ndfa in either harvest, values from these two legume species were combined into a single ureide calibration, *RU-N* (%) = 0.5661 *x %Ndfa* + 0.6688. *M. pruriens* was a weak ureide producer in the glasshouse, with low (<20%) stem RU-N in both harvests, while field measurements showed RU-N up to 28%. The glasshouse calibration for this species was clearly not adequate for field use in PNG and so the *P. phaseoloides*/*C. mucunoides* calibration was also applied to the single *M. pruriens* field sampling. In the glasshouse it appeared that *M. pruriens* was less sensitive to nitrate since it was still fixing >25% of its N at 10 mM nitrate while *P. phaseoloides* and *C. mucunoides* fixed <5% of their total plant N. Nitrate reduced N_2 fixation in all three legume species as observed in the reduced %Ndfa and nodulation (total nodules/nodule mass/total N). In all three legume species, nitrate increased the shoot and root N concentrations (%N) in the two harvests. An interesting observation was that nitrate did not affect the N concentrations (%N) in the nodules of all three species in both harvests, possibly because N in nodules came directly from N_2 fixation irrespective of available soil nitrate. A similar reduction in %Ndfa with increasing soil nitrate was observed in field sampling in PNG but it was difficult to relate nodulation to soil nitrate in the field because nodule dry weight was not measured and a subjective

scoring system was used. Overall, the growth of *M. pruriens* was excellent in the glasshouse with significant increases in root and shoot dry weights and total N (g/plant) with increased nitrate. *P. phaseoloides* and *C. mucunoides* had growth problems that resulted in some poor responses to nitrate, especially in total N (g/plant) and plant biomass.

The calibrated ureide technique was applied successfully in the oil palm plantations in West New Britain Province in Papua New Guinea for the first time to estimate legume dependence on N_2 fixation. The ureide technique measures N_2 fixation at a point in time only and not necessarily for the lifespan of legumes so the %Ndfa may change over time in the legume species. However, some studies (Alves *et al.* 2000a; Peoples *et al.* 1996) have shown that similar %Ndfa values were obtained from the ureide technique and ^{15}N-based techniques (Unkovich *et al.* 2008) in perennial legumes during specific growth periods. The ureide calibration in the current study also provides a basis for future N_2 fixation studies to be undertaken. Nitrogen fixation in the legume standing biomass plus litter averaged 8.3 kg N/ha across the different plantations (2 to 25 year old), with a maximum of 34 kg N/ha. Unfortunately the time period that the standing biomass and litter accumulated over is not measured. However, if one were to use the measure of N_2 fixed in the 2 year old plantation sampled (34.2 kg N/ha/2 years), then at least 17.1 kg of N/ha/year might be fixed by legumes. Total root N could constitute around 30% of total plant N (Unkovich *et al.* 2010a); adding this value to the above estimation would increase it to an average of 22 kg N fixed/ha/year. The legume biomass production time was not accounted for, which could be less than 1 year or 6 months, and if measured, this could increase the total fixed N/ha/year. It is recommended that future work should estimate the legume biomass production time. Other work suggests that the standing shoot biomass production (144 – 443 g/m^2) obtained in the current field study is similar to 3-4 months of legume growth (Fosu 2003; Franke *et al.* 2008; Giller 2001a; Sanginga 2003; Sanginga *et al.* 1996a; Sanginga *et al.* 1996b; Sylvester-Bradley 1984; Tian *et al.* 1999). If so, then the amount of N fixed estimated in this study as 22 kg N/ha/year could actually represent what was fixed within 3 to 4 months; the total N fixed annually under PNG oil palm plantations could then be 3 to 4 times higher than this (66 to 88 kg N/ha/year). It should be noted that this amount was calculated at 44% of legume ground cover while other estimates of N fixed/ha/year were estimated with legume cover taken at 100%. For example, Zahara et al (1986) extrapolated the amount of N fixed by *P. phaseoloides* in g/m^2 to kg/ha by direct conversion with no reference to the % ground cover in a 2 year old oil palm plantation

(Broughton 1977; Zaharah *et al.* 1986). Oil palm trees occupy roughly 1% of the area in a hectare of plantation and this could be taken out of the calculation as well. Further research is warranted to quantify the legume biomass production time when estimating N_2 fixation in order to know the amount of N fixed/ha/year. Litter, on the other hand, accumulates within 6 months of growth in *P. phaseoloides* (Vesterager *et al.* 1995), which may suggest that the standing litter biomass estimation in the current study could have accumulated over 18 months since the litter dry matter is 3 times higher than the standing shoot biomass.

In the fields currently surveyed in West New Britain, an average of 124 kg N/ha was applied as fertilizer in 2011 in 2 to 6 year old plantations. If we use the 2006 fertilizer costs (US$1.20/kg N) (Banabas 2007) converted at the current exchange rate (PNGK1 = US$0.4357), a total of US$151 worth of ammonium nitrate fertilizers would be required to fertilize one hectare of oil palm field. If we substitute some of the required mineral N with the estimated N fixed/ha/year (17 kg N/ha/year), and apply the estimate to 0 to 6 year old plantations (since legume ground cover in these plantation ages would be similar), a value of US$20.40 could be saved per hectare. If more than 2000 hectares of oil palm plantations are within 0 to 6 year old every year, an expected equivalent of US$40, 000 could be saved every year if legume N fixation is maintained at 17 kg N/ha/year. On the other hand, if the same amount of N fixed is achieved in all plantation ages (current company-owned plantations occupy over 85,700 ha, (RSPO 2012)), then more than US$1.5 million could be saved every year. This amount of N fixed/ha/year could be higher depending on the true estimates of N_2 fixation. It is therefore recommended that future research be undertaken to quantify the root N contribution, the legume biomass production time, the legume shoot/litter mineralisation rate and where possible, the eventual uptake of fixed N by the palms, so that the total N balance from legumes could be well understood and easily integrated into oil palm management. The major advantage of legume use compared to mineral fertilizers is that legume litter mineralises at a slower rate so that not much nitrate would be available or lost at once through leaching, although concentrations of NO_3 up to 24 mg/kg of soil were observed in the present study, whose origin (either from legume litter mineralisation or applied nitrate) is unknown. The longer time it takes for legume litter to mineralise, the longer N remains in the system which could potentially benefit the oil palm.

A key finding in the field survey was that legume cover was low (mean of 9.8% ground cover) and it was the primary determinant of low N fixation. Unrelated to this was a

decrease in legume shoot dry matter (g/m^2) with plantation age which was attributed to increasing shade as the oil palm canopy closed, although no measurements have been done to quantify this. A useful model could be developed relating stand age to legume total N and available NO_3 to %Ndfa; together these could be used to construct an empirical model of N_2 fixation under oil palm in PNG. Such approaches have been used for legumes elsewhere, such as chickpea in Australia (Herridge *et al.* 1998). While this may work for the system in West New Britain, such relationships should not be extrapolated beyond the region in which they were developed (Unkovich *et al.* 2010a). However, the ureide calibrations may be usefully applied elsewhere.

Some management effort might be necessary to increase legume cover in all plantation ages if N fixation inputs are required. It was noted in the survey that N_2 fixation decreased with age, but the dependence of legumes on N fixation did not necessarily decrease with the age of oil palms. *C. caeruleum* under the older plantations (>15 years old) was still fixing >50% of its total N. This is important to know so that the use of *C. caeruleum* and other shade tolerant species could be maximised under older plantations.

In conclusion, this study has shown that the relatively simple and inexpensive xylem ureide analysis can be calibrated in a glasshouse and applied in the field to estimate proportional dependence on N fixation for the tropical cover legumes *P. phaseoloides* and *C. mucunoides*, although there may be difficulties using it for *M. pruriens*. This presents a valuable tool for assessing inputs of fixed N in PNG oil palm systems, particularly since the natural abundance method could not be applied in West New Britain oil palm plantations. Further glasshouse studies could also be undertaken for all three species and *C. caeruleum* under more optimal temperature conditions to clarify growth, N accumulation and N fixation responses of each legume species to nitrate, and determine if the ureide calibration will be similar for the two Calapogonium species *C. caeruleum* and *C. mucunoides*. An important finding was that soil nitrate reduced N_2 fixation in the field legumes as was seen in the glasshouse experiment as well. This may highlight an opportunity to reduce N fertiliser inputs to the oil palm system because N fixation may be able to make up for some of the shortfall. Practices such as applying the mineral N fertilizers in the frond piles and other areas of the plantation like the weeded circle instead of on legume cover plants might also be worth exploring. Further field sampling with more replicates to measure stem RU-N, percentage legume cover, legume biomass production over a given time and legume

litter mineralisation studies are recommended. The two latter activities are especially important because informed decisions could be made on the timing when legumes could substitute for mineral N fertilizers in oil palm plantations. This will contribute to the effective management of nutrients and achieve a sustainable oil palm production in PNG.

6 Appendices

Appendix 1: Amounts of allantoin, asparagin (Asp) + glutamine (Gln) and nitrate used with distilled water to make up standard curves for a) ureide, b) amino and c) nitrate respectively

a) Ureide

Concentration (mM)	1.0 mM allantoin (mL)	Distilled water (mL)
0.00	0.00	10.0
1.25	0.5	9.5
2.50	1.0	9.0
5.00	2.0	8.0
10.0	4.0	6.0
15.0	6.0	4.0

b) Amino

Concentration (mM)	2.0 mM Asp/Gln (mL)	Distilled water (mL)
0.00	0.00	10.0
0.01	0.5	9.5
0.02	1.0	9.0
0.04	2.0	8.0
0.10	5.0	5.0

c) Nitrate

Concentration (mM)	25 mM nitrate (mL)	Distilled water (mL)
0.00	0.00	10.0
1.25	0.5	9.5
2.50	1.0	9.0
5.00	2.0	8.0
10.0	4.0	6.0
15.0	6.0	4.0

Appendix 2: Effect of nitrate on nodule numbers between different legume species in the a) first and b) second harvests.
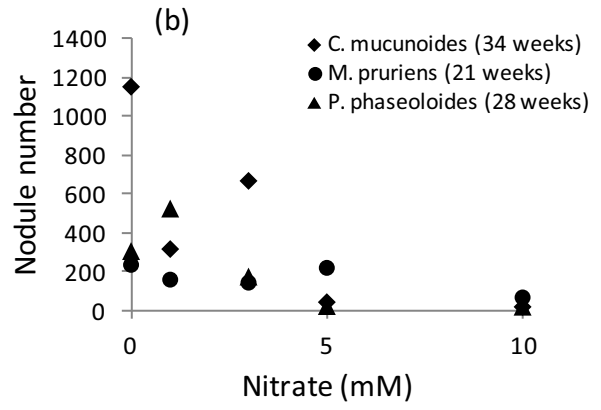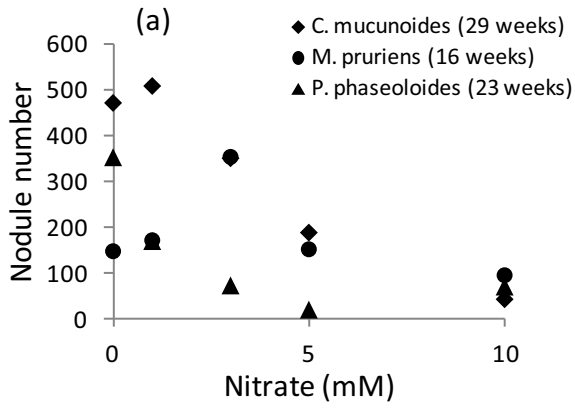
Appendix 3: Statistical analyses on the effect of nitrate on nodule number, stem relative ureide-N (%) and percentage of N derived from the atmosphere (%Ndfa) in a) first and b) second harvests of C. mucunoides, M. pruriens and P. phaseoloides

a) Harvest 1

Nitrate (mM)	C. mucunoides (29 weeks)			M. pruriens (16 weeks)			P. phaseoloides (23 weeks)		
	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)
0	470.0	92.5	100.0	145.8	13.5	100.0	351.3	70.3	100.0
1	507.0	45.9	68.9	169.8	13.6	89.2	168.0	29.9	69.0
3	349.0	27.5	37.8	352.0	18.7	69.4	71.0	12.1	15.7
5	186.5	17.4	16.5	150.0	13.3	46.2	18.0	7.1	4.2
10	40.8	5.3	-0.6	93.3	3.9	33.0	68.5	5.4	4.9
	NS	P<0.001	P<0.001	P=0.038	NS	P<0.001	P=0.002	P<0.001	P<0.001
Grand mean	311.0	37.7	44.5	182.0	12.6	67.6	135.0	25.0	38.8
LSD$_{0.05}$		21.8	22.5	163.1		14.7	146.0	19.5	12.2
cv%		38.4	33.6	59.4		14.4	71.6	51.7	20.9

b) Harvest 2

Nitrate (mM)	Calopo (34 weeks)			M. pruriens (21 weeks)			P. phaseoloides (28 weeks)		
	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)	Nodule number	Stem RU-N (%)	%Ndfa (^{15}N)
0	1149.5	34.7	100.0	232.3	10.7	100.0	303.3	66.2	100.0
1	313.5	35.3	56.4	156.0	9.5	85.7	522.3	14.8	80.1
3	664.8	3.2	46.9	140.5	2.4	58.2	171.8	9.9	25.1
5	40.0	1.7	3.5	216.8	2.0	44.0	20.0	3.9	4.7
10	15.8	1.4	0.8	64.3	1.8	18.2	16.8	2.3	2.3
	NS	P=0.005	P<0.001	P=0.05	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001
Grand mean	437.0	15.3	41.5	162.0	5.3	61.2	207.0	19.4	42.4
LSD$_{0.05}$		22.6	31.4	115.8	3.1	7.6	204.6	17.5	10.1
cv%		98.1	50.2	47.4	38.4	8.2	65.7	59.6	15.8

Appendix 4: A two-way ANOVA carried out to observe the effect of nitrate on the grand means of nodule numbers between two harvests in each legume species with significance levels at P≤0.05.

Nitrate (mM)	C. mucunoides		M. pruriens		P. phaseoloides	
	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2
0	470	1150	146	232	351	303
1	507	314	170	156	168	522
3	349	665	352	141	71	172
5	187	40	150	217	18	20
10	41	16	93	64	69	17
Mean	311	437	182	162	135	207
Harvest	NS		NS		NS	
Harvest x N treatment	NS		**P=0.028**		**P=0.009**	
$LSD_{0.05}$			**136.0**		**170.0**	
CV%			**54.5**		**68.9**	

Appendix 5: Correlation analyses on the percentage of N derived from the atmosphere (%Ndfa) and nodule mass in M. pruriens in the a) first and b) second harvests as a comparison to the correlation done on the nodule number in Figure 5.

(a) *M. pruriens* (16 weeks)

$R^2 = 0.8087$

(b) *M. pruriens* (21 weeks)

$R^2 = 0.0252$
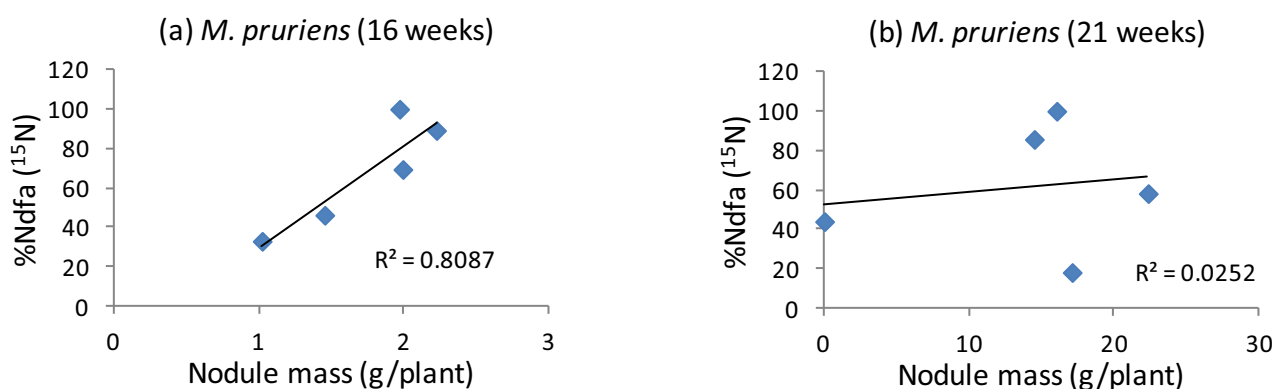
Appendix 6: Mean dry weight (grams/plant) of nodules, root and shoot of C. mucunoides, M. pruriens and P. phaseoloides when fed with different amounts of nitrate in a) harvest 1 and b) harvest 2, and the difference between the grand means of each legume species' plant dry weights with significance levels at P≤0.05.

(a) Harvest 1

Nitrate (mM)	C. mucunoides (29 weeks)				M. pruriens (16 weeks)				P. phaseoloides (23 weeks)			
	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant
0	0.7	2.7	10.4	13.8	2.0	2.4	17.1	21.5	1.3	3.1	13.7	18.1
1	0.7	3.0	13.2	17.0	2.2	2.6	20.5	25.2	0.5	1.6	7.1	9.2
3	0.4	4.8	16.0	21.2	2.0	2.6	28.8	33.4	0.3	1.7	8.2	10.1
5	0.2	3.2	14.5	17.9	1.5	3.3	25.5	30.3	0.1	0.9	4.1	5.0
10	0.1	3.6	22.4	26.0	1.0	3.4	32.0	36.5	0.3	4.7	26.1	31.0
Av plant dry wt (g)				19.2				29.4				14.7
Grand mean	NS	NS	NS	NS	P=0.009	NS	P=0.014	P=0.028	P=0.001	P=0.023	P=0.008	P=0.01
	0.4	3.5	15.3	19.2	1.7	2.9	24.8	29.4	0.5	2.4	11.8	14.7
LSD0.05					0.7		8.7	9.5	0.5	2.3	11.6	14.1
cv%					25.1		23.2	21.5	68.9	64.2	64.9	63.4

GM: P<0.001, 21.1 LSD0.05: 7.0 cv%: 52.5

(b) Harvest 2

Nitrate (mM)	Calopo (34 weeks)				M. pruriens (21 weeks)				P. phaseoloides (28 weeks)			
	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant
0	1.0	5.2	18.1	24.3	4.8	7.8	34.1	46.7	1.6	4.4	17.9	23.9
1	0.2	2.6	6.5	9.2	4.8	9.3	52.8	67.0	2.1	5.8	24.9	32.8
3	0.6	6.3	24.7	31.6	2.1	7.1	35.1	44.3	0.5	4.1	16.4	21.0
5	0.0	1.9	9.5	11.4	2.5	9.7	51.4	63.6	0.0	1.7	7.0	8.7
10	0.0	2.6	18.4	20.9	1.0	7.1	28.1	36.2	0.0	1.7	10.6	12.3
Av plant dry wt (g)				19.5				51.6				19.7
Grand mean	NS	NS	NS	NS	P<0.001	NS	P=0.042	P=0.033	P<0.001	NS	P=0.035	P<0.029
	0.4	3.7	15.4	19.5	3.1	8.2	40.3	51.6	0.9	3.5	15.3	19.7
LSD0.05					1.3		18.6	21.2	0.8		11.3	15.1
cv%					28.5		30.7	27.3	61.7		48.7	50.8

GM: P<0.001, 30.3 LSD0.05: 10.5 cv%: 55.0

Appendix 7: Mean of total N (grams/plant) in nodules, roots and shoot of C. mucunoides, M. pruriens and P. phaseoloides when fed with nitrate in a) harvest 1 and b) harvest 2 with significance levels at P≤0.05.

a) Harvest 1: Total N in plants

Nitrate (mM)	C. mucunoides (29 weeks)				M. pruriens (16 weeks)				P. phaseoloides (23 weeks)			
	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant
0	0.04	0.05	0.29	0.39	0.12	0.06	0.49	0.67	0.06	0.05	0.42	0.53
1	0.04	0.06	0.41	0.52	0.12	0.06	0.60	0.78	0.02	0.04	0.24	0.30
3	0.02	0.11	0.53	0.66	0.11	0.06	0.75	0.92	0.01	0.06	0.28	0.34
5	0.01	0.08	0.56	0.65	0.08	0.09	0.50	0.66	0.00	0.04	0.22	0.26
10	0.00	0.09	1.00	1.09	0.05	0.10	0.88	1.04	0.01	0.14	0.91	1.07
	NS	NS	NS	NS	P=0.009	P=0.007	P=0.01	P=0.037	P=0.008	P=0.005	P=0.002	P=0.003
Grand mean	0.02	0.08	0.56	0.66	0.10	0.07	0.64	0.81	0.02	0.07	0.41	0.50
$LSD_{0.05}$					0.04	0.03	0.23	0.27	0.03	0.05	0.32	0.39
cv%					28.0	23.4	23.7	21.9	92.2	54.4	51.0	51.9

b) Harvest 2: Total N in plants

Nitrate (mM)	Calopo (34 weeks)				M. pruriens (21 weeks)				P. phaseoloides (28 weeks)			
	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant	Nodules	Roots	Shoot	Plant
0	0.07	0.12	0.45	0.64	0.20	0.14	0.44	0.78	0.07	0.08	0.44	0.59
1	0.01	0.05	0.14	0.20	0.19	0.16	0.74	1.09	0.09	0.10	0.60	0.79
3	0.03	0.15	0.74	0.92	0.09	0.15	0.66	0.91	0.02	0.08	0.42	0.52
5	0.00	0.05	0.44	0.49	0.11	0.25	1.16	1.52	0.00	0.04	0.28	0.32
10	0.00	0.08	0.79	0.87	0.04	0.24	0.82	1.11	0.00	0.05	0.51	0.56
	NS	NS	NS	NS	P<0.001	P<0.001	P=0.011	P=0.017	P<0.001	NS	NS	NS
Grand mean	0.02	0.29	0.51	0.63	0.13	0.19	0.77	1.08	0.04	0.07	0.45	0.56
$LSD_{0.05}$					0.05	0.05	0.36	0.41	0.03			
cv%					25.0	17.2	31.4	25.2	63.1			

Appendix 8: The results of a two-way ANOVA carried out to show the effect of nitrate on the dry weights of nodules, roots, shoot and plant total weights between two different harvests for a) C. mucunoides, b) M. pruriens and c) P. phaseoloides with significance levels at P≤0.05.

a) *C. mucunoides*

Nitrate (mM)	Nodule wt (g)		Roots wt (g)		Shoot wt (g)		Plant wt (g)	
	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2
0	0.7	1.0	2.7	5.2	10.4	18.1	13.8	24.3
1	0.7	0.2	3.0	2.6	13.2	6.5	17.0	9.2
3	0.4	0.6	4.8	6.3	16.0	24.7	21.2	31.6
5	0.2	0.0	3.2	1.9	14.5	9.5	17.9	11.4
10	0.1	0.0	3.6	2.6	22.4	18.4	26.0	20.9
Mean	0.4	0.4	3.5	3.7	15.3	15.4	19.2	19.5
Harvest	NS		NS		NS		NS	
$LSD_{0.05}$	0.5		-		-		-	
Harvest x N treatment	NS		NS		NS		NS	
$LSD_{0.05}$	-		-		-		-	
CV%	125.9		72.9		88.4		85.1	

b) *M. pruriens*

Nitrate (mM)	Nodule wt (g)		Roots wt (g)		Shoot wt (g)		Plant wt (g)	
	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2
0	2.0	4.8	2.4	7.8	17.1	34.1	21.5	46.7
1	2.2	4.8	2.6	9.3	20.5	52.8	25.2	67.0
3	2.0	2.1	2.6	7.1	28.8	35.1	33.4	44.3
5	1.5	2.5	3.3	9.7	25.5	51.4	30.3	63.6
10	1.0	1.0	3.4	7.1	32.0	28.1	36.5	36.2
Mean	1.7	3.1	2.9	8.2	24.8	40.3	29.4	51.6
Harvest	**P<0.001**		**P<0.001**		**P<0.001**		**P<0.001**	
$LSD_{0.05}$	**0.4**		**0.983**		**6.22**		**7.05**	
N Treatment	**P<0.001**		NS		NS		NS	
$LSD_{0.05}$	**0.7**							
Harvest x N treatment	**P<0.001**		NS		**P=0.005**		**P=0.004**	
$LSD_{0.05}$	**1.0**				**13.9**		**15.8**	
CV%	**28.8**		27.5		**29.6**		**27**	

c) *P. phaseoloides*

Nitrate (mM)	Nodule wt (g)		Roots wt (g)		Shoot wt (g)		Plant wt (g)	
	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2	Harvest 1	Harvest 2
0	1.3	1.6	3.1	4.4	13.7	17.9	18.1	23.9
1	0.5	2.1	1.6	5.8	7.1	24.9	9.2	32.8
3	0.3	0.5	1.7	4.1	8.2	16.4	10.1	21.0
5	0.1	0.0	0.9	1.7	4.1	7.0	5.0	8.7
10	0.3	0.0	4.7	1.7	26.1	10.6	31.0	12.3
Mean	0.5	0.9	2.4	3.5	11.8	15.3	14.7	19.7
Harvest	**P=0.01**		NS		NS		NS	
LSD$_{0.05}$	**0.291**							
N Treatment	**P<0.001**		NS		**P=0.019**		**P=0.021**	
LSD$_{0.05}$	**0.5**				**7.73**		**9.89**	
Harvest x N treatment	**P=0.003**		**P=0.009**		**P=0.003**		**P=0.003**	
LSD$_{0.05}$	**0.7**		**2.7**		**10.9**		**14.0**	
CV%	**67**		**62.6**		**55.8**		**56.2**	

Appendix 9: Percentages of nitrate, amino and ureides in the xylem sap of M. pruriens at different rates of nitrate in a) first and b) second harvests

(a) 16 weeks

(b) 21 weeks
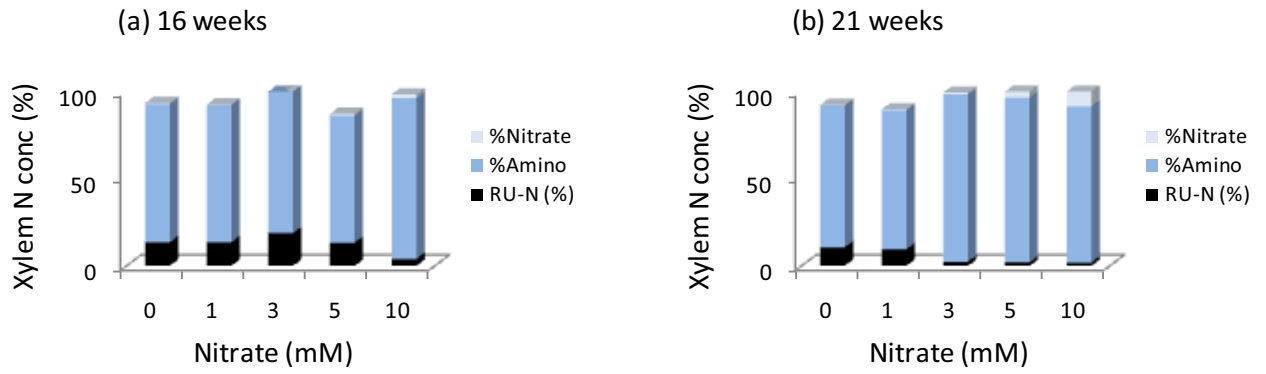
Appendix 10: Correlation analyses between shoot and litter N concentrations with the age of palms.
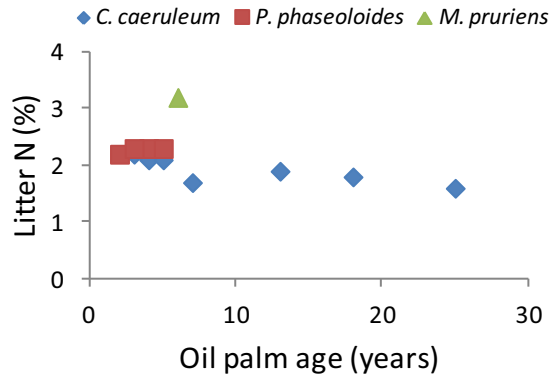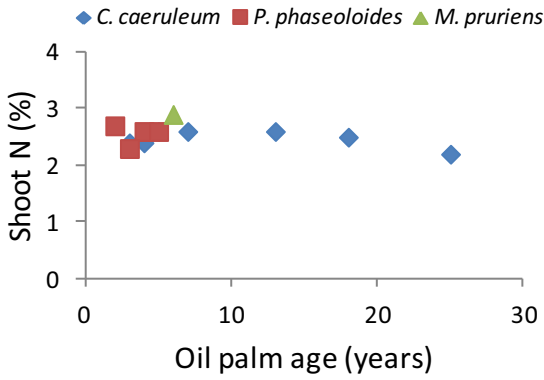
Appendix 11: Correlation analyses between legume stem RU-N and %Ndfa with age of palms

7 References

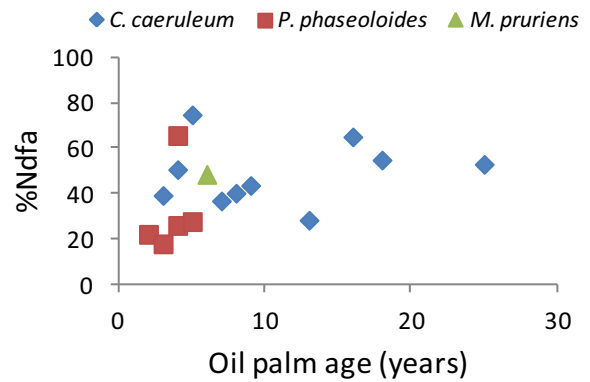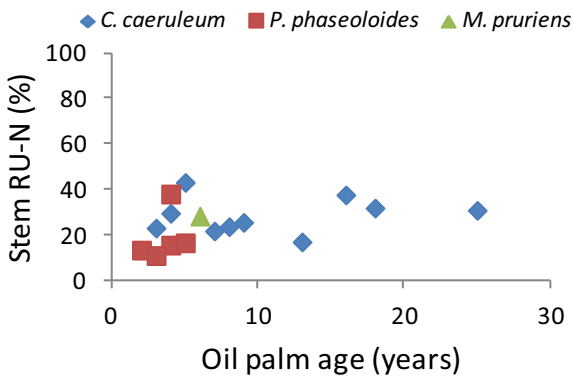Agamuthu P, Broughton WJ (1985) Nutrient cycling within the developing oil palm-legume ecosystem. *Agriculture, Ecosystems & Environment* **13**, 111-123.

Agamuthu P, Chan YK, Jesinger R, Khoo KM, Broughton WJ (1981) Effect of differently managed legumes on the early development of oil palms (*Elaeis guineensis* Jacq.). *Agro-Ecosystems* **6**, 315-323.

Alves BJR, Resende AS, Rezende CdP, Macedo R, Tarré RM, Urquiaga S, Boddey RM (2000a) Estimation of N_2 fixation in *Desmodium ovalifolium* from the relative ureide abundance of stem solutes: comparison with the ^{15}N-dilution and an in situ soil core technique. *Nutrient Cycling in Agroecosystems* **56**, 177-193.

Alves BJR, Resende AS, Urquiaga S, Boddey RM (2000b) Biological nitrogen fixation by two tropical forage legumes assessed from the relative ureide abundance of stem solutes: ^{15}N calibration of the technique in sand culture. *Nutrient Cycling in Agroecosystems* **56**, 165-176.

Banabas M (2007) Study of nitrogen loss pathways in oil palm (*Elaeis guineensis* Jacq.) growing agro-ecosystems on volcanic ash soils in Papua New Guinea. Massey University, New Zealand

Barker AV, Bryson GM (2007) Nitrogen. In 'Handbook of Plant Nutrition.' (Eds AV Barker and DJ Pilbeam). (Taylor & Francis Group: United States of America)

Barker AV, Pilbeam DJ (Eds) (2007) 'Handbook of Plant Nutrition.' (Taylor & Francis Group: United States of America)

Becker M, Johnson DE (1999) The role of legume fallow in intensified upland rice-based systems of West Africa. *Nutrient Cycling in Agroecosystems* **53**, 71-81.

Blackmer TM, Schepers JS, Varvel GE, Walter-Shea EA (1996) Nitrogen deficiency detection using reflected shortwave radiation from irrigated corn canopies. *Agronomy Journal* **88**, 5.

Boddey RM, Knowles R (1987) Methods for quantification of nitrogen fixation associated with Gramineae. *Critical Reviews in Plant Sciences* **6**, 209 - 266.

Boddey RM, Peoples MB, Palmer B, Dart PJ (2000) Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody perennials. *Nutrient Cycling in Agroecosystems* **57**, 235-270.

Boddey RM, Urquiaga S, Neves MCP, suhet AR, Peres J (1990) Quantification of the contribution of N_2 fixation to field-grown grain legumes - a strategy for the practical application of the ^{15}N isotope dilution technique. *Soil Biology and Biochemistry* **22**, 649-655.

Bolger T, Pate J, Unkovich MJ, Turner N (1995) Estimates of seasonal nitrogen fixation of annual subterranean clover-based pastures using the ^{15}N natural abundance technique. *Plant and Soil* **175**, 57-66.

Broughton WJ (1976a) Effect of various covers on soil fertility under Hevea brasiliensis muell. arg. and on growth of the tree. *Agro-Ecosystems* **3**, 147-170.

Broughton WJ (1976b) Effect of various covers on soil fertility under *Hevea brasiliensis* muell. arg. and on growth of the tree. *Agro-Ecosystems* **3**, 147-170.

Broughton WJ Effect of various covers on the performance of *Elaeis guineensis* (Jacq.) on different soils. . In '*International Developments in Oil Palm. Malaysian International Agricultural Oil Palm Conference.*', 14-17 June 1976 1977, Kuala Lumpur. (Eds DA Earp and W Newall) ISP, pp. 501-525

Burns RC, Hardy RWF (1975) 'Nitrogen fixation in bacterial and higher plants.' (Springer-Verlag Berlin Heidelberg: New York)

Cadisch G, Schunke RM, Giller KE (1994) Nitrogen cycling in a pure grass pasture and a grass-legume mixture on a red latosol in Brazil. *Tropical Grasslands* **28**, 43-52.

Cadisch G, Sylvester-Bradley R, Nösberger J (1989) ^{15}N-Based estimation of nitrogen fixation by eight tropical forage-legumes at two levels of P:K supply. *Field Crops Research* **22**, 181-194.

Carranca C, de Varennes A, Rolston D (1999) Biological nitrogen fixation by fababean, pea and chickpea, under field conditions, estimated by the ^{15}N isotope dilution technique. *European Journal of Agronomy* **10**, 49-56.

Cataldo D, Maroon M, Schrader L, Youngs V (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil Science & Plant Analysis* **6**, 71-80.

Caudwell RW (2000) The successful development and implementation of an integrated pest management system for oil palm in Papua New Guinea. *Integrated Pest Management Reviews* **5**, 297-301. [In English]

Ciompi S, Gentili E, Guidi L, Soldatini GF (1996) The effect of nitrogen deficiency on leaf gas exchange and chlorophyll fluorescence parameters in sunflower. *Plant Science* **118**, 177-184.

Corley RHV, Tinker PB (2008) 'The Oil Palm, Fourth Edition.' (Wiley-Blackwell)

Couteaux M-M, Bottner P, Berg B (1995) Litter decomposition, climate and liter quality. *Trends in Ecology & Evolution* **10**, 63-66.

Daimon H, Yoshioka M (2001) Responses of root nodule formation and nitrogen fixation activity to nitrate in a split-root system in peanut (*Arachis hypogaea* L.). *Journal of Agronomy & Crop Science* **187**, 89-95.

Dawson T, Brooks PD (2001) Fundamentals of stable isotope chemistry and measurement In 'Application of stable isotope techniques to study biological processes and functioning of ecosystems.' (Eds MJ Unkovich, JS Pate, A McNeill and DJ Gibbs) pp. 1-18. (Kluwer Academic: Dordrecht)

Dilworth M, Glenn A (1984) How does a legume nodule work? *Trends in Biochemical Sciences* **9**, 519-523.

Dilworth MJ, Glenn AR (Eds) (1991) 'Biology and biochemistry of nitrogen fixation.' (Elsevier Science Publishers B. V. : Amsterdam)

Duthion C, Pigeaire A (1993) Stability of the nitrogen concentration of inoculated white lupins during pod development. *Annals of Botany* **72**, 55-61.

Erisman JW, Bleeker A, Galloway J, Sutton MS (2007) Reduced nitrogen in ecology and the environment. *Environmental Pollution* **150**, 140-149. [In English]

Escuredo PR, Minchin FR, Gogorcena Y, Iturbe-Ormaetxe I, Klucas RV, Becana M (1996) Involvement of activated oxygen in nitrate-induced senescence of pea root nodules. *Plant Physiology* **110**, 1187-1195.

Fairhurst T, Hardter R (Eds) (2003) 'Oil palm: Management for large and sustainable yields (First edn).' (IPNI and IPI: Singapore)

FAOSTATS (2013) Food and Agriculture Organisation of the United Nations. In. ' (FAO Statistics Division: Italy)

Fosu M (2003) Nitrogen accumulation and release by Sunn Hemp, Calopo, Mucuna and devil bean in semi-arid Ghana. *Agricultural and Food Science Journal of Ghana* **2**, 141-153.

Frank JS (Ed.) (1982) 'Nitrogen in agricultural soils.' (American Society of Agronomy: Wisconsin USA)

Franke A, Laberge G, Oyewole BD, Schulz S (2008) A comparison between legume technologies and fallow, and their effects on maize and soil traits, in two distinct environments of the West African savannah. *Nutrient Cycling in Agroecosystems* **82**, 117-135.

Fujita K, Ofosu-Budu KG, Ogata S (1992) Biological nitrogen fixation in mixed legume-cereal cropping systems. *Plant and Soil* **141**, 155-175.

Galloway JN (1998) The global nitrogen cycle: changes and consequences. *Environmental Pollution* **102**, 15.

Gil JL, Guenni O, Espinoza Y (1997) Biological N_2-fixation by three tropical forage legumes and its transfer to *Brachiaria humidicola* in mixed swards. *Soil Biology and Biochemistry* **29**, 999-1004.

Giller KE (2001a) Legumes as green manures and cover crops. In 'Nitrogen Fixation in Tropical Cropping Systems.' Ed. KE Giller). (CAB International Publishing)

Giller KE (2001b) 'Nitrogen fixation in tropical cropping systems ' 2nd edn. (CABI Publishing: London)

Giller KE, Fairhurst T (2003) Legume cover plants. In 'Oil palm: management for large and sustainable yields.' 1st edn. (Eds T Fairhurst and R Hardter) pp. 384. (Potash & Phosphate Institute (PPI)/Potash: Singapore)

Giller KE, Wilson KJ (1991) 'Nitrogen fixation in tropical cropping systems.' (C.A.B International: London)

Gruber N, Galloway JN (2008) An Earth-system perspective of the global nitrogen cycle. *Nature* **451**, 293-296. [In English]

Hansen AP (1994) 'Symbiotic N_2 fixation of crops leugmes, achievements and perspectives.' (Druckerei Engelhardt, Neunkirchen: Germany)

Henderson J, Osborne DJ (2000) The oil palm in all our lives: how this came about. *Endeavour* **24**, 63-68.

Henzell E, Martin A, Ross P, Haydock K (1968) Isotopic studies on the uptake of nitrogen by pasture plants. IV. Uptake of nitrogen from labelled plant material by Rhodes grass and Siratro. *Australian Journal of Agricultural Research* **19**, 65-77.

Herridge DF (1982) Use of the ureide technique to describe the nitrogen economy of field-grown soybeans. *Plant Physiology* **70**, 7-11.

Herridge DF (1984) Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum extracted exudates of soybean. *Crop Science* **25**, 173-179.

Herridge DF, Marcellos H, Felton W, Turner G, Peoples MB (1998) Chickpea in wheat based cropping systems of northern New South Wales III. Prediction of N_2 fixation and N balance using soil nitrate at sowing and chickpea yield. *Australian Journal of Agricultural Research* **49**, 409-418.

Herridge DF, Palmer B, Nurhayati DP, Peoples MB (1996) Evaluation of the xylem ureide method for measuring N_2 fixation in six tree legume species. *Soil Biology and Biochemistry* **28**, 281-289.

Herridge DF, Peoples MB (1990) Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. *Plant Physiology* **93**, 495-503.

Herridge DF, Peoples MB (2002a) Calibrating the xylem-solute method for nitrogen fixation measurement of ureide-producing legumes: cowpea, mungbean, and black gram. *Communications in Soil Science & Plant Analysis* **33**, 425.

Herridge DF, Peoples MB (2002b) Timing of xylem sampling for ureide analysis of nitrogen fixation. *Plant and Soil* **238**, 57-67.

Herridge DF, Peoples MB, Boddey R (2008) Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil* **311**, 1-18.

Hungria M, Vargas MAT (2000) Environmental factors affecting N_2 fixation in grain legumes in the tropics, with an emphasis on Brazil. *Field Crops Research* **65**, 151-164.

Koczberski G, Curry G, Gibson K (2001) 'Improving productivity of the smallholder oil palm sector in Papua New Guinea: a socio-economic study of the Hoskins and Popondetta schemes.' (The Australian National University: Canberra) 233

Koutika LS, Hauser S, Henrot J (2001) Soil organic matter assessment in natural regrowth, *Pueraria phaseoloides* and *Mucuna pruriens* fallow. *Soil Biology and Biochemistry* **33**, 1095-1101.

Lensi R, Lescure C, Steinberg C, Savoie J-M, Faurie G (1991) Dynamics of residual enzyme activities, denitrification potential, and physico-chemical properties in a γ-sterilized soil. *Soil Biology and Biochemistry* **23**, 367-373.

Lucinski R, Polcyn W, Ratajczak L (2002) Nitrate reduction and nitrogen fixation in symbiotic association *Rhizobium* - legumes. *Acta Biochimica Polonica* **49**, 537-546.

Malik KA, Naqvi SHM, Aleem MIH (Eds) (1985) 'Nitrogen and the environment.' (Nuclear Institute for Agriculture and Biology: Pakistan)

McClure PR, Israel DW, Volk RJ (1980) Evaluation of the relative ureide content of xylem sap as an Indicator of N_2 fixation in soybeans greenhouse studies. *Plant Physiology* **66**, 720-725.

McNamara NP, Black HIJ, Beresford NA, Parekh NR (2003) Effects of acute gamma irradiation on chemical, physical and biological properties of soils. *Applied Soil Ecology* **24**, 117-132.

Melillo JM, Aber JD, Muratore JF (1982) Nitrogen and lignin control of hardwood leaf litter decomposition dynamics. *Ecology* **63**, 621-626.

Mendham DS, Kumaraswamy S, Balasundaran M, Sankaran KV, Corbeels M, Grove TS, O'Connell AM, Rance SJ (2004) Legume cover cropping effects on early growth and soil nitrogen supply in eucalypt plantations in south-western India. *Biology and Fertility of Soils* **39**, 375-382.

Nelson P, Webb MJ*, et al.* (2010) 'Environmental sustainability of oil palm cultivation in Papua New Guinea.' (ACIAR: Canberra)

Nieder R, Benbi DK (2008) 'Carbon and nitrogen in the terrestrial environment.' (Springer Science + Media B. V.: Netherlands)

Omena-Garcia RP, Justino GC, Sodek L, Goncalves JFdC (2011) Mineral nitrogen affects nodulation and amino acid xylem transport in the Amazonian legume *Inga edulis* Mart. *International Jornal of Plant Physiology and Biochemistry* **3**, 215-218.

Onwu AC, Ayuba SA, Ali A (2009) Contribution of leguminous weed (*Calopogonium mucunoides*) to soil fertility in Makurdi, Southern Guinea savanna of Nigeria. *Journal of Sustainable Agriculture and the Environment* **11**, 75-82. [In English]

Orrell I, Rees Hv*, et al.* (2009) 2008 Annual Reseach Report. PNG Oil Palm Research Association Inc., Port Moresby.

Pate JS, Atkins CA, White ST, Rainbird RM, Woo KC (1980) Nitrogen nutrition and xylem transport of nitrogen in ureide-producing grain legumes. *Plant Physiology* **65**, 961-965. [In English]

Pate JS, Dart PJ (1961) Nodulation studies in legumes. *Plant and Soil* **15**, 329-346.

Pennington JA, VanDevender K, Jennings JA (2009) Nutrient and fertilizer value of dairy manure. In 'Agriculture and Natural Resources. ' pp. 5. (University of Arkansas: Arkansas)

Peoples MB, Brockwell J*, et al.* (2009a) The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* **48**, 1-17.

Peoples MB, Craswell ET (1992) Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. *Plant and Soil* **141**, 13-39.

Peoples MB, Hebb DM, Gibson AH, Herridge DF (1989) Development of the xylem ureide assay for the measurement of nitrogen fixation by pigeonpea (*Cajanus cajan* (L.) Millsp.). *Journal of Experimental Botany* **40**, 535-542.

Peoples MB, Herridge DF (1990) Nitrogen fixation by legumes in tropical and subtropical agriculture. In 'Advances in Agronomy. Vol. Volume 44.' Ed. NC Brady) pp. 155-223. (Academic Press)

Peoples MB, Palmer B, Lilley DM, Duc LM, Herridge DF (1996) Application of ^{15}N and xylem ureide methods for assessing N_2 fixation of three shrub legumes periodically pruned for forage. *Plant and Soil* **182**, 125-137.

Peoples MB, Unkovich MJ, Herridge DF (2009b) Measuring symbiotic nitrogen fixation by legumes. In 'Nitrogen fixation in crop production.' Ed. DW Emerich, Krishnan, H. B.). (American Society of Agronomy: United States of America)

Pongwichian P, Clermont-Dauphin C, Dissataporn C, Suvannang N (2010) A qualitative assessment of the N status of young rubber trees as affected by interrank crops in Northeast Thailand. In '19th

World Congress of Soil Science, Soil Solutions for a Changing World. ' pp. 10-13: Brisbane, Australia)

Purcell LC, Serraj R, de Silva M, Sinclair TR, Bona S (1998) Ureide concentration of field-grown soybean in response to drought and the relationship to nitrogen fixation. *Journal of Plant Nutrition* **21**, 949-966.

Pypers P, Verstraete S, Thi CP, Merckx R (2005) Changes in mineral nitrogen, phosphorus availability and salt-extractable aluminium following the application of green manure residues in two weathered soils of South Vietnam. *Soil Biology and Biochemistry* **37**, 163-172.

Ramos MG, Villatoro MAA, Urquiaga S, Alves BJR, Boddey RM (2001) Quantification of the contribution of biological nitrogen fixation to tropical green manure crops and the residual benefit to a subsequent maize crop using ^{15}N-isotope techniques. *Journal of Biotechnology* **91**, 105-115.

Rayment GE, Lyons DJ (2011) 'Soil chemical methods Australasia.' (CSIRO: Melbourne) 495

Reiter K, Schmidtke K, Rauber R (2002) Estimation of symbiotic N$_2$ fixation by a low-level, large-scale ^{15}N application technique. *Soil Biology and Biochemistry* **34**, 303-314.

Rennie R, Rennie D, Siripaibool C, Chaiwanakupt P, Boonkerd N, Snitwongse P (1988) N$_2$ fixation in Thai soybeans: effect of tillage and inoculation on1^{5}N-determined N$_2$ fixation in recommended cultivars and advanced breeding lines. *Plant and Soil* **112**, 183-193.

Reynolds SG (1982) Contributions to yield, nitrogen fixation and transfer by local and exotic legumes in tropical grass-legume mixtures in Western Samoa. *Tropical Grasslands* **16**, 76-80.

RSPO (2010) Promoting the growth and use of sustainable palm oil. In. ' (Roundtable on Sustainable Palm Oil: Kuala Lumpur)

RSPO (2012) An international multistakeholder organisation and certification scheme for sustainable palm oil. In. Vol. 2013'. (Roundtable on Sustainable Palm Oil: Switzerland)

Salako FK, Olowokere FA, Tian G, Kirchhof G, Osiname O (2007) Ground cover by three crops cultivated on marginal lands in southwestern Nigeria and implications for soil erosion. *Spanish Journal of Agricultural Research* **5**, 497-505.

Sanford P, Pate JS, Unkovich MJ (1994) A survey of proportional dependence of subterranean clover and other pasture legumes on N$_2$ fixation in south-west Australia utilizing ^{15}N natural abundance. *Australian Journal of Agricultural Research* **45**, 165-181.

Sanford P, Pate JS, Unkovich MJ, Thompson AN (1995) Nitrogen fixation in grazed and ungrazed subterranean clover pasture in south-west Australia assessed by the ^{15}N natural abundance technique. *Australian Journal of Agricultural Research* **46**, 1427-1443.

Sanginga N (2003) Role of biological nitrogen fixation in legume based cropping systems; a case study of West Africa farming systems. *Plant and Soil* **252**, 25-39.

Sanginga N, Ibewiro B, Houngnandan P, Vanlauwe B, Okogun JA, Akobundu IO, Versteeg M (1996a) Evaluation of symbiotic properties and nitrogen contribution of mucuna to maize grown in the derived savanna of West Africa. *Plant and Soil* **179**, 119-129.

Sanginga N, Okogun A, Akobundu IO, Carsky RJ, Tian G, Wirkom LE (1996b) Nodulation and estimation of symbiotic nitrogen fixation by herbaceous and shrub legumes in Guinea savanna in Nigeria. *Biology and Fertility of Soils* **23**, 441-448.

Schweiger P, Hofer M, Hartl W, Wanek W, Vollmann J (2012) N_2 fixation by organically grown soybean in Central Europe: method of quantification and agronomic effects. *European Journal of Agronomy* **41**, 11-17.

Shelton HM, Stur WW (Eds) (1990) 'Forages for plantation crops
Proceedings of a workshop.' ACIAR Proceedings (Australian Centre for International Agricultural Reseach: Canberra)

Sorensen P, Weisbjerg MR, Lund P (2003) Dietary effects on the composition and plant utilization of nitrogen in dairy cattle manure. *Journal of Agricultural Science - Cambridge* **141**, 79-92.

Streeter JG (1982) Synthesis and accumulation of nitrite in soybean nodules supplied with nitrate. *Plant Physiology* **69**, 1429-1434.

Streeter JG (1985) Nitrate inhibition of legume nodule growth and activity. *Plant Physiology* **77**, 321-324.

Streeter JG, Wong PP (1988) Inhibition of legume nodule formation and N_2 fixation by nitrate. *Critical reviews in plant sciences* **7**, 1-23.

Suvannang N, Clermont-Dauphin C, Cheylan V, Promratrak K, Ninchawee C, Sakonnakhon SPN (2010) Decomposition of cover crop residues in a rubber tree plantation in northeast Thailand. In '19th World Congress of Soil Science, Soil Solutions for a Changing World. ' Brisbane, Australia)

Sylvester-Bradley R (1984) Rhizobium inoculation trials designed to support a tropical forage legume selection programme. *Plant and Soil* **82**, 377-386.

Sylvester-Bradley R, Mosquera D, Asakawa NM, Sanchez G (1991) Promiscuity and responses to rhizobial inoculation of tropical kudzu (*Pueraria phaseoloides*). *Field Crops Research* **27**, 267-279.

Thomas RJ, Asakawa NM (1993) Decomposition of leaf litter from tropical forage grasses and legumes. *Soil Biology and Biochemistry* **25**, 1351-1361.

Tian G, Kang BT (1998) Effects of soil fertility and fertilizer application on biomass and chemical compositions of leguminous cover crops. *Nutrient Cycling in Agroecosystems* **51**, 231-238.

Tian G, Kolawole GO, Salako FK, Kang BT (1999) An improved cover crop-fallow system for sustainable management of low activity clay soils of the tropics. *Soil Science* **164**, 671-682.

Unkovich MJ, Baldock J, Peoples MB (2010a) Prospects and problems of simple linear models for estimating symbiotic N_2 fixation by crop and pasture legumes. *Plant and Soil* **329**, 75-89.

Unkovich MJ, Baldock J, Peoples MB (2010b) Prospects and problems of simple linear models for estimating symbiotic N 2 fixation by crop and pasture legumes. *Plant and Soil* **329**, 75-89.

Unkovich MJ, Herridge DF, Peoples MB, Cadisch G, Boddey B, Giller KE, Alves BJR, Chalk P (2008) 'Measuring plant-associated nitrogen fixation in agricultural systems.' (Australian Centre for International Agricultural Research (ACIAR): Canberra)

Unkovich MJ, Pate JS (2000) An appraisal of recent field measurements of symbiotic N2 fixation by annual legumes. *Field Crops Research* **65**, 211-228.

Vernon M (1978) Macroclimate the lignin control of litter decomposition rates. *Ecology* **59**, 465-472.

Vesterager JM, Osterby S, Jensen ES, Schjoerring JK (1995) Symbiotic N-2-fixation by the cover crop Pueraria phaseoloides as influenced by litter mineralization. *Plant and Soil* **177**, 1-10. [In English]

Viera-Vargas MS, Souto CM, Urquiaga S, Boddey RM (1995) Quantification of the contribution of N_2 fixation to tropical forage legumes and transfer to associated grass. *Soil Biology and Biochemistry* **27**, 1193-1200.

Vitousek PM, Cassman K, *et al.* (2002) Towards an ecological understanding of biological nitrogen fixation. *Biogeochemistry* **57-58**, 1-45.

Voisin A-S, Salon C, Munier-Jolain NG, Ney B (2002) Quantitative effects of soil nitrate, growth potential and phenology on symbiotic nitrogen fixation of pea (<i>Pisum sativum</i> L.). *Plant and Soil* **243**, 31-42.

Wani SP, Rupela OP, Lee KK (1995) Sustainable agriculture in the semi-arid tropics through biological nitrogen fixation in grain legumes. *Plant and Soil* **174**, 29-49.

Werner D, Newton WE (Eds) (2005) 'Nitrogen fixation in agriculture, forestry, ecology, and the environment.' Nitrogen fixation: origins, applications, and research progress (Springer: The Netherlands)

Yanggen D, Reardon T (2001) Kudzu-improved fallows in Peruvian Amazon. In 'Agricultural Technologies and Tropical Deforestation.' (Eds A Angelsen and D Kaimowitz). (CAB International 2001)

Yemm EW, Cocking EC (1955) The determination of amino-acids with ninhydrin *Analyst* **80** 209-214.

Yoneyama T, Nambiar PTC, Lee KK, Srinivasa Rao B, Williams JH (1990) Nitrogen accumulation in three legumes and two cereals with emphasis on estimation of N2 fixation in the legumes by the natural 15N-abundance technique. *Biology and Fertility of Soils* **9**, 25-30. [In English]

Young EG, Conway CF (1942) On the estimation of allantoin by the Rimini-Schryver reaction. *Journal of Biological Chemistry* **142**, 839-853.

Zaharah AR, Sharifuddin HAH, Razley MN, Mohd Saidi AK (1986) Measurement of nitrogen fixed by *Pueraria phaseoloides* by N-15 dilution technique. *Pertanika* **9**, 45-49.

Zahran HH (1999) Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiology and Molecular Biology Reviews* **63**, 968-989.

Zehr JP, Montoya JP (2007) Measuring N2 Fixation in the Field. In 'Biology of the Nitrogen Cycle.' (Eds B Hermann, JF Stuart and EN William) pp. 193-205. (Elsevier: Amsterdam)

Zotarelli L, Zatorre NP, Boddey RM, Urquiaga S, Jantalia CP, Franchini JC, Alves BJR (2012) Influence of no-tillage and frequency of a green manure legume in crop rotations for balancing N outputs and preserving soil organic C stocks. *Field Crops Research* **132**, 185-195.