LINCOLN
UNIVERSITY
Te Whare Wānaka O Aoraki

PO Box 84 Lincoln University, Canterbury, New Zealand
Telephone No:(64)(03)325-2811
Fax No:(64)(03)325-3843

JCDww

Department of Plant Science

11 April 1996

This is to certify that the work described in this thesis was planned, executed and described by JOSEPHINE WANJIKU under our direct supervision.

Dr Don Mead
Supervisor

Prof. Kuan Goh
Associate Supervisor

Dr Ruth Gadgil
Associate Supervisor

BIOLOGICAL NITROGEN FIXATION OF SOME LEGUMES AT A COASTAL SAND DUNE FOREST SITE IN NEW ZEALAND

A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Master of Applied Science

at

Lincoln University

by

J. Wanjiku

Lincoln University

1996

Abstract of a thesis submitted in partial fulfilment of the

requirements for the Degree of M. Appl. Sci.

BIOLOGICAL NITROGEN FIXATION OF SOME LEGUMES AT A COASTAL SAND DUNE FOREST SITE IN NEW ZEALAND

By J. Wanjiku

Field experiments were conducted at Woodhill Forest, near Auckland for a period of one year (July 1994 to July 1995), in a clearfelled, first-rotation replanted *Pinus radiata* stand. The study was aimed at screening three legumes namely: Maku lotus (*Lotus pedunculatus* Cav. "Grasslands Maku"), hairy canary clover (*Dorycnium hirsutum* (L) Ser.), and everlasting pea (*Lathyrus latifolius* L.) (Experiment 1), as possible replacement for the yellow tree lupin (*Lupinus arboreus* Sims.). The seasonal biological N_2 fixation (BNF) of the legumes was monitored using the ^{15}N isotope dilution technique and the acetylene reduction assay (ARA). Biological N_2 fixation was also monitored in an operational area sown with a mixture of serradella (*Ornithopus sativus* Brot. "Grasslands koha") and *Lotus pedunculatus* (Experiment 2). Sampling was carried out in winter (July) and spring (November) of 1994, and summer (February) and winter (July) of 1995.

Dorycnium showed highest biomass which did not differ significantly between seasons. This species also accumulated the highest dead dry matter yield. The accumulation was probably due to its relatively low N concentration in the dead dry matter (0.6% N) compared to those of *Lotus* and *Lathyrus* (1.3 and 1.4% N respectively).

Dorycnium and *Lathyrus* derived on average, 98 and 95% of their annual N from the atmosphere respectively, which was not significantly affected by season. *Lotus* on the other hand showed a significantly lower proportion of nitrogen derived from the atmosphere (%Ndfa) between spring and summer than between winter and spring. This was probably a result of high inorganic soil N derived from the decomposing plant material as well as the high temperatures and dry conditions experienced during summer, all of which led to low biological N_2 fixation (BNF).

Using the ^{15}N isotope dilution technique, *Lathyrus* was estimated to have fixed significantly high annual amounts of N (214 kg N ha^{-1} yr^{-1}), compared to 55, 71 and 133 kg N ha^{-1} yr^{-1}, fixed by *Lotus*, *Dorycnium* and legumes in experiment 2, respectively. The annual amounts of N_2 fixed by *Lotus* and *Dorycnium* were not significantly different. For all the legumes, most of the N_2 was fixed in the period between winter and spring probably due to more dry matter yield increment and the favourable weather conditions during spring. Due to its high N_2 fixation, low accumulation of dead dry matter and high dry matter increment, *Lathyrus* seems the most suitable species for the replacement of the lupin.

In both experiments no N was found to have accumulated in the mineral soil after 3-4 years of legume growth. The variability in such a site and the difficult in the detection of total N makes N accumulation studies unsuitable.

The ARA as applied in this study showed trends that could only be explained on the basis of the prevailing weather conditions during the assay. In most cases the method also showed lower N_2 fixation values when compared to the ^{15}N method. The under-estimation of N_2 fixation in the ARA was probably due to errors introduced as a result of extrapolation after a short period of incubation, diurnal variations in light, temperature and moisture in the period prior to sampling and the failure to have the conditions of the assay matching carefully with those of N_2 fixation at the time of sampling. The ^{15}N isotope dilution technique was better placed for field estimation of N_2 fixation due to its ability to give integrated values.

Key words: Legumes, *Lotus*, *Dorycnium*, *Lathyrus*, *Ornithopus*, Biological nitrogen fixation (BNF), ^{15}N isotope dilution technique, acetylene reduction assay (ARA), N accumulation, sand dunes.

CONTENTS

List of Tables

List of Figures

List of Appendices

CHAPTER 1

GENERAL INTRODUCTION

The productivity of temperate forest ecosystems is limited in most cases by the lack of available nitrogen (N) which has led to the use of inorganic fertilisers to sustain production (Gordon, 1984; McColl and Edmonds, 1986; Thomas and Mead, 1992a). Economic advantage is often achieved by the application of inorganic N, but sometimes this type of operation is uneconomic (Baker *et al.* 1986) and inefficient given that trees recover less than 30% of the applied N (Mead and Pritchett, 1975; Thomas and Mead, 1992b). The remainder of the fertiliser is either retained, immobilised within the soil, or lost from the ecosystem through leaching and denitrification (Baker *et al* 1986; Thomas and Mead, 1992b). This indicates unacceptable leakage of N to ground water and low recoveries (Mead and Pritchett 1975; Ballard and Will, 1978; Ziehm *et al.* 1992). For New Zealand, estimates showed that if all the responsive exotic forestry were to be treated with fertiliser, 14,000 tonnes N would have been required in 1985 (Ballard and Will, 1978). This fertiliser level, due to economic reasons, would have been difficult to achieve (Will, 1981). Efforts are now being made to utilise biologically fixed N as a means of providing cheaper and more sustainable N supply to forests (Nambiar and Nethercott, 1987; Zeihm *et al.* 1992) by using forage legumes (Goodman, 1988; Zeihm *et al.* 1992) or leguminous trees (Danso *et al.* 1992).

Most forest management interferes with N accumulation and recycling processes that develop in any undisturbed ecosystem. Few practices increase N supply in the ecosystem, yet many have the potential of removing N from the forest site (Will *et al.* 1980; Trowbridge and Holl, 1992). In New Zealand, there are a few managed forests where N is not a growth limiting factor at some stage in rotation (Will, 1978). Nitrogen

deficiency reduces tree diameter more than the height (Will and Hodgkiss, 1977), thus reducing the harvestable tree volume to a large extent.

Though legumes may add the required N to an ecosystem, they have been reported to compete with trees for nutrients, moisture and light and thus depress tree growth (Cole and Newton, 1986; Mansur, 1994). Legumes selected as understorey should be able to benefit the plantation by suppressing unwanted weeds that may compete with trees in the early stage of tree growth (Cole and Newton, 1986; Nambiar, 1990), increase the soil total N and in turn promote tree growth (Haines *et al.* 1978; Nambiar and Nethercott, 1987).

The radiata pine forests on the coastal sand dunes of New Zealand in the past depended on the perennial yellow tree lupin (*Lupinus arboreus* Sims) for much of their N supply. Sand dune stabilisation, using the artificial succession of marram grass/tree lupin/radiata pine resulted in a successful production forestry operation, especially at Woodhill Forest (Gadgil *et al.* 1984). It proved difficult to establish lupin without the preliminary stabilisation with marram grass and impossible to establish young pine trees without the shelter of the lupins. Exclusion of lupins from the ecosystem resulted in lowered productivity of the pines due to slow growth rates associated with symptoms of N deficiency (Gadgil, 1976; Gadgil *et al.* 1984; Beets and Madgwick, 1988).

The lupin plants at Woodhill regenerated naturally after each thinning and harvesting operation and populations were, until recently, self sustaining once established in an area. The sudden appearance of lupin blight caused by the fungus *Colletotrichum gloeosporioides* (Penzig), first recognised in 1989 (Williams, 1993 Dick, 1994), and its spread throughout New Zealand, has necessitated a search for alternative nitrogen fixing plants. It is hoped that the alternative species will grow with the marram grass and compete with other plants in the ecosystem so as to produce

continuous cover and to supply the required N. The present study aims to monitor the symbiotic nitrogen fixation of some legumes at Woodhill in an attempt to screen them as possible replacement of the lupins.

The objectives of the present study were to:-

1. estimate the amount of atmospheric N_2 fixed by the legumes as influenced by season;
2. compare the ^{15}N dilution technique and acetylene reduction assay as methods of estimating N_2 fixation in the field;
3. estimate the amount of N accumulated in legume and non-legume plots since legume establishment.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Biological nitrogen fixation (BNF) has great potential for maintaining and increasing forestry productivity (Gordon, 1983; Nambiar and Nethercott, 1987; Ziehm *et al.* 1992). Production of *Pinus radiata* on the coastal sand dune forests of New Zealand, basically limited by N, has been made possible by provision of N from lupins. Recently formed sand dunes in this site contain 0.008% N, most of which is unavailable for plant growth (Gadgil, 1983). The presence of lupins in this ecosystem increased N availability in the soil thus reducing the requirement for artificial fertiliser application. Gadgil, (1971b), estimated that the lupins could fix 160 kg N ha^{-1} yr^{-1} in the lupin/marram stand at Woodhill forest.

Lupin plants naturally declined and died out under low light conditions (canopy closure) and thus did not act as a serious competitor to the tree crop. The present death of the lupin is thus a big concern since the once stabilised sand dune may once again be exposed to wind erosion due to declined vigour of the marram grass. It is also likely that the older forest stands forest will stagnate, young stands will be less productive and there is an increased risk of reversion to drifting sand dune in exposed areas. There is thus an urgent need to screen other nitrogen fixing legumes as a replacement for the lupin in order to maintain sand dune forestry and to protect the inland farms from encroaching sand.

This chapter reviews the role of legumes in forest ecosystems, factors that affect biological nitrogen fixation (BNF) and methods used to estimate BNF in the field.

2.2. Sand dune stabilisation procedure

2.2.1. Artificial succession technique

New Zealand is long and narrow, with an area of 26.5 million hectares lying between 34° and 47°S (Wendelken, 1974). About 50,000 ha of coastal sand in the North Island pose great threat to farm lands since the foredunes are exposed to the prevailing westerly winds (Sprent and Silvester, 1973; Berg and Smithies, 1973). Stabilisation and afforestation of the shifting sand dune have the objective of halting the sand drift and reclaiming land for pastoral and agricultural purposes (Wendelken, 1974). To stabilise this mobile sand an artificial succession technique was developed; this begins with constructing foredune barriers to cut off the supply of sand to the inland areas as well as to break the force of winds at ground level in the immediate vicinity of the foredune (Restall, 1964; Wendelken, 1974; Gadgil, 1979). Marram grass (*Ammophilia arenaria* (L) Link), an effective sand binding species, is then planted into the sand dune ecosystem, and its establishment adds 3 kg N ha^{-1} to the mobile sand (Gadgil, 1971b). After one year of marram growth, the sand is sufficiently stabilised for the yellow tree lupin (*Lupinus arboreus* Sims) to be introduced by direct seeding between grass tussocks (FRI, 1977; van Kraayenoord, 1986).

2.2.2. Role of lupin in the coastal sand dune forests of New Zealand

Lupin plants usually provided sufficient cover for the pine trees to be planted 2-4 years after seed sowing. By the time of pine tree planting, about 446 kg N had accumulated in the ecosystem (Gadgil, 1983). The lupins and the marram grass were tractor crushed when the pine trees were introduced to reduce competition for the first 6-12 months (Gadgil, 1971a). Later the lupins were hormone sprayed to release the pine trees. At the start of the second and subsequent rotations, and after thinning, the lupins

continued to provide additional N supplies, thus reducing the requirement for the application of artificial fertiliser to the N-poor soils. The lupin was highly successful in this role because of its ability to thrive in the difficult environment of a wind swept west coast due to its deep rooting system and non-palatability to animals (Gadgil, 1977; Gadgil *et al.* 1986; Dick, 1994). It was also a prolific seed producer and the seeds could survive in the soil for many years until soil disturbance and appropriate conditions stimulated germination, making the lupin self sustaining once established in the area (Dick, 1994).

2.3. Biological nitrogen fixation (BNF) in forest ecosystems

2.3.1. Role of biological nitrogen fixation in forests

The primary interest in BNF has been amelioration of the inherent low N status of many forests and to address losses caused by some management practices. This in turn should improve the growth of the non-N_2-fixing forest crop which benefits from the fixed N (Ta and Faris, 1987). Legumes in forest management should provide 50-100 kg N ha^{-1} yr^{-1} for 3-5 years to give an economic return (Jorgensen, 1980). This amount will also ensure some response by most conifers during and in the year immediately after the conifer establishment (Turvey and Smethurst, 1983).

An N_2 fixing understorey may also be important, not only in N cycling, but for other nutrients as well. It may serve as a source and sink of available nutrients thus reducing losses from litter and soil layers (Outcalt and White, 1979).

The N_2 fixing plants could also improve soil organic matter, soil structure and cation exchange capacity (CEC) (Sprent, 1983). Rapid establishment of a leguminous cover crop after clear-felling could also reduce run off and soil movement, and may

temporarily immobilise nutrients that could be lost from the site following rapid rate of mineralisation of soil organic matter (Turvey and Smethurst, 1980).

The use of wide initial tree spacing and low final crop stocking of radiata pine in New Zealand forestry has increased the potential for weed growth in the forest stands (Sutton, 1976; Gadgil *et al.* 1988). As an alternative to herbicide treatment, the concept of replacing weeds with less competitive, easily controlled or even useful understorey is attractive. Weed control cost would be saved, provided the understorey does not inhibit tree crop growth by competing for moisture in critical periods (Turvey and Smethurst, 1980). *Lupinus arboreus* had been successful in the sand dune ecosystem since it grows faster than most of the developing weed population (Gadgil *et al.* 1986). Alternative legumes to the yellow tree lupins will thus be successful if they can outgrow the weeds in the ecosystem that may take up and immobilise the fixed N.

2.3.2. Limitation to use of BNF

The use of N_2 fixing plants as an alternative to fertiliser N in exotic forest is attractive, but poses problems to forest managers. The typical vigorous growth of these plants means that the plantation trees must be protected from competition during the first year of establishment and the extra cost for minimising competition, perhaps in the form of herbicide use, must be met. There is also the opportunity cost of land occupied by a non-commercial crop, plus the potentially increased fire hazard from the understorey. Later in the rotation the legumes plants are unlikely to realise their potential unless tree stands remain relatively open grown or are sufficiently thinned for understorey to thrive (Will *et al.* 1980; Hendrickson and Burgess, 1989). Another disadvantage of using N_2 fixers in forestry is related to the cost of establishment and supplying minerals or inoculum essential for successful root nodulation (Turvey and

Smethurst, 1980). Most legumes need more P, S, and Mo than other plants if they are to fix N (FRI, 1981).

Biological N_2 fixing legumes derive part of their nitrogen from the atmosphere and a portion from the soil (LaRue and Patterson, 1981). For forest use it would be productive to select legumes that derive most of their nitrogen from the atmosphere. This could maximise the benefits of N accretion to the site while minimising the potential competition for soil N between the legumes and the tree crop (Schoeneberger *et al.* 1989).

Legumes may also compete with the tree crop for moisture, light and space and thus depress tree growth (Cole and Newton, 1986; Mansur, 1994). Mansur (1994) working in a silvopastoral system at Lincoln University found that the foliar N status of the pine was occasionally reduced by pasture treatments. Lucerne (*Medicago sativa* L.) was found to pose severe competition to the trees; it reduced the tree growth and occasionally reduced the tree foliar N content. Thus appropriate legumes to use as intercrops in forest plantations should increase the trees' total N through N transfer (Haines *et al.* 1978; Nambiar and Nethercott, 1987).

The primary limit to the use of BNF is that the technology is currently more complex than the use of chemical fertilisers. It also has a perceived lower financial rate of return in comparison with application of chemical fertiliser (Gordon, 1984). For instance, for most legumes it is not known how much N_2 is fixed and therefore the economic returns can not be quantified (Turvey and Smethurst, 1983). Also for most legume-tree combinations it is not known how the legumes perform under the shade of the tree crop, nor how they compete with other plants so that the ecosystem can benefit from the N_2 fixed.

Turvey and Smethurst (1980), proposed a biological criteria for selection of N_2 fixing understorey. The likelihood that any N_2 fixing plant would meet the criteria can only be judged from assessment made under specific climate/soil/silviculture combinations for which it is required (Gadgil *et al.* 1986).

2.4. Factors affecting biological nitrogen fixation

The nitrogen fixing potential (NFP) of a species is the amount of N_2 fixed with all the environmental constraints removed (Danso *et al.* 1992). Thus the actual N_2 fixed is dictated by the plants nitrogen fixing potential and the environmental factors (Sprent *et al.* 1988).

The range and magnitude of temperature experienced at soil surface may limit the occurrence of susceptible rhizobia and may limit their number within the host plant (Vincent, 1974). High and low temperature have depressive effects on nodule formation and N_2 fixation, although a variable range can be tolerated by rhizobia (Lie, 1974). Temperate legumes can tolerate as low as 7°C, while tropical ones can withstand 20°C. McColl and Edmonds (1986), found that the rate of fixation decreases abruptly with higher temperatures, above 30°C, but less abruptly below 20°C. Temperatures affect N_2 fixation through the disturbance of plant metabolism (Lie, 1981), root formation (Rohini-Kumarasighe and Nutman, 1979), and rhizobia growth and infection.

Most rhizobia multiplication will be favoured by moist but not water-logged conditions (Sprent and Silvester, 1973; Sprent, 1973; Vincent, 1974; Sprent, 1979; Lie, 1981). Water stress reduces photosynthesis as a source of energy for fixation and transpiration and induces the formation of abnormal root hairs, which are not suitable for nodule formation (Lie, 1981). Water-logging limits the availability of oxygen for

root metabolism and growth (McColl and Edmonds, 1986). Moisture contributes most to seasonal variation in nitrogenase activity (O'Connell and Grove, 1987).

Nodule formation and N_2 fixation are strongly reduced in the presence of moderate to high levels of combined nitrogen (Lie, 1974; Peoples *et al.* 1989a). Combined nitrogen when supplied to the nodulated plant, is taken up readily by the plant with a reduction in N_2 fixation. Soils low in initial mineral N will have low legume production if there is failure to achieve symbiosis (Kanehiro *et al.* 1983). In soils with higher mineral N the legumes may compensate for poor N_2 fixation by taking up N from the soil. Thus cropping with a legume deficient in nodulation is likely to exploit the N reserves (Peoples *et al.* 1989a). In establishment of some plantation forests, fertilisers are required. These fertilisers contain the elements most limiting to tree growth and these will probably be the same element limiting growth of the understorey legume. Thus fertilisers may also be necessary for the establishment of the legumes in order to enable them achieve the biological nitrogen fixing potential (Turvey and Smethurst, 1983).

In acid soils enhanced plant uptake of manganese (Mn) and aluminium (Al) may tend towards toxic concentrations. Also minerals, such as calcium (Ca) and molybdenum (Mo) essential for symbiosis are less available in such soils (Sprent, 1979; McLaren and Cameron, 1990; Ledgard *et al.* 1995). Both of these factors affect rhizobia and nodulation, in turn inhibiting N_2 fixation.

Nitrogen fixation also varies considerably within and between seasons due to plant age and environmental effects on plant growth. During the first season when plant growth is exponential, BNF increases with time. As the plant ages the N stored in roots and stem may reduce BNF by a feed back mechanism (Danso *et al.* 1992).

2.5. Mechanisms of nitrogen transfer from legumes to trees

As legumes fix N_2 for their own use, they normally release little N into the soil while growth is occurring (Ta and Faris, 1987). Decomposition and mineralisation of dead plant organs are the major pathways for N transfer in the absence of animals (Saratchandra and Upsdell, 1981; Ta and Faris, 1987). Animals may excrete over 80% of the N ingested from the legume fodder (Haynes and Williams, 1993), providing an efficient function of transfer from their urine which contains mostly urea N and is readily available for use by the trees. However, leaching losses of N from urine patches are likely to be high as soil N levels are well in excess of plant requirements (Ball, 1982; Ledgard and Saunders, 1982).

Ledgard and Saunders (1982) supported the possibility that the major N transfer in the field, happens due to senescence. In this case, the factors that affect plant senescence which include plant defoliation, soil moisture, temperature and light (Evan, 1973; Saratchandra and Upsdell, 1981) affect N transfer. It is possible that both defoliation and shading reduce photosynthesis and carbohydrate supply to roots. Defoliation may cause small quantities of amino acids, ammonia and nitrate to be released from the roots. Instead of transporting N from the roots to the shoots, it is secreted in the rhizosphere because of lack of enough photosynthates. Temperature and moisture mostly affect the rate of mineralisation and in optimum conditions the legume roots decompose rapidly (Whitehead *et al.* 1979; Berg *et al.* 1987).

In soils where the leaching and denitrification losses are likely, it is important that the fixed N should be retained until it can be utilised by the trees. In young stands retention might be achieved by the introduction of a third suitable but non competing species. The trees assuming total dominance at the time of maximum demand and

maximum soil exploitation would manipulate this source of N supply to advantage (Mead and Gadgil, 1978).

Transfer of N from legumes to the soil N pool may occur by direct excretion from the legume roots (Ledgard *et al.* 1985; Ta and Faris, 1987). Haystead *et al.* (1988) observed that vesicular arbuscular mycorrhizal (VAM), hyphal strands help in translocating nitrogen from the legumes to the non-legume. A direct transfer of nitrogen may occur in such a situation because of direct translocation along hyphae links.

2.6. Methods of estimating biological nitrogen fixation

2.6.1. Introduction

The future management of legumes will require accurate knowledge on the amounts of N_2 fixed by crops in the field (LaRue and Patterson, 1981). Accurate measurement of BNF is important in order to assess the overall benefits in a forestry system (Peoples and Herridge, 1990). Four methods; the N-difference method, N-fertiliser equivalence, acetylene reduction assay (ARA) and the ^{15}N isotope methods are commonly used to estimate N_2 fixation in the field (Peoples *et al.* 1989a; Peoples and Herridge, 1990). Other methods less commonly used include the ureide and nodule evaluation methods.

2.6.2. Ureide Method

Xylem sap carries N-containing compounds from the roots to the shoots of field grown legumes. These compounds are assimilation products of N_2 fixed or soil mineral N taken up by the roots (Peoples and Herridge, 1990). The first stable product of N_2 fixation in the legume nodule is ammonia. There is a substantial difference in the principal forms of N transported in the xylem between highly symbiotic and

unnodulated plants. It is thus possible to use the abundance of ureides related to the N compounds in the xylem sap as an indirect measure of the proportion of plant N derived from the N_2 fixed (Herridge *et al.* 1990; Giller and Wilson, 1991). The method offers simple and reliable procedures for evaluating the symbiotic performance of supernodulating plants (Hansen *et al.* 1989).

Limitations of this technique include the difficulty in extraction of the sap. To carry out ureide assay, xylem sap is obtained by decapitating plants and collecting the bleeding sap from the cut (Herridge and Peoples, 1989; Giller and Wilson ,1991). Other limitations include variation in composition of the sap with plant age (Peoples *et al.* 1989b). It also provides only a short-term measure of symbiosis (Peoples and Herridge, 1990). The method can only be used in legumes that transport products of N_2 fixation as ureides.

2.6.3. N-fertiliser Equivalence

This method assesses the amount of N_2 fixed by growing N fertilised non-N_2-fixing plants in plots alongside unfertilised N_2 fixing test legume plants. The N fertiliser level at which the yields of the non-N_2-fixing plants match those of the legume is equivalent to the amount of N_2 fixed. The value obtained is usually expressed as fertiliser N-equivalence (LaRue and Patterson, 1981; Peoples and Herridge, 1990).

Differences in N-fertiliser use efficiency and losses of fertiliser through volatilisation, denitrification and leaching make direct comparisons of experiments in different regions difficult (Peoples and Herridge, 1990).

2.6.4. Nodule Evaluation

Since N_2 fixation is dependent on the formation and maintenance of nodules, the degree of nodulation as determined by nodule number, weight or size, or by the subjective rating for colour and distribution in the rooting system has been used as a measure of symbiotic activity (Peoples *et al.* 1989a). Nodule evaluation is quick, convenient and inexpensive.

Nodule assessment can at best provide an indication of legume potential to fix N_2, but it cannot be used to quantify the amount of N_2 fixed (Peoples and Herridge, 1990). It can assist in data evaluation and interpretation since poor nodulation is often the basis for poor N_2 fixation.

2.6.5. N-difference Method

This method is used to compare N yield of a legume with that of non-legume (LaRue and Patterson, 1981). The amount of N_2 fixed is calculated by subtracting N yield of the non-legume from that of the legume. The difference is assumed to be the N derived by BNF.

The basic assumption is that the legume derives all its N from the atmosphere, which results in over-estimation of N_2 fixation (Peoples and Herridge, 1990). Other processes such as extraction of N by roots from soil horizons and differences due to root morphology and growth pattern can affect the N-uptake (LaRue and Patterson, 1981). Other assumptions in this technique are that the N contained in the non-N_2-fixing control plants is derived only from the soil N and that the legume and the control crops assimilate the same amounts of soil N (Peoples and Herridge, 1990).

2.6.6. Acetylene reduction assay (ARA)

2.6.6.1. Principles of ARA

The acetylene reduction assay (ARA) arose from observations that the N_2-fixing complex nitrogenase also catalyses the reduction of acetylene (C_2H_2) to ethylene (C_2H_4) (Gallon and Chaplin, 1987; Peoples and Herridge, 1990). Standard ARA methods involve enclosing detached nodules or nodulated root systems in airtight containers and exposing them to an atmosphere containing C_2H_2 (Witty and Minchin, 1988). After an incubation period, gas samples are collected and analysed for ethylene production using gas chromatography (Turner and Gibson, 1980). The calculation of the amount of N_2 fixed involves application of a ratio to relate the amount of C_2H_2 reduced to N_2 fixed (LaRue and Patterson, 1981; Peoples and Herridge, 1990).

2.6.6.2. Advantages of ARA

Some advantages of ARA include the fact that the method is substantially simpler than other methods since the product is retrieved readily as a gas sample which can be analysed directly. The *in situ* assay process and the gas chromatography equipment are relatively portable, simple and inexpensive (Hardy *et al.* 1973, Turner and Gibson, 1980; Danso *et al.* 1992). The assay is widely applicable and easy to perform (Vessey, 1994), making it more attractive than the other methods (Turner and Gibson, 1980; Witty and Minchin, 1988).

2.6.6.3. Problems associated with the ARA

A major limitation of ARA lies in the difficulty of converting the assay values to absolute amounts of N_2 fixed (Ruegg and Alston, 1978). The theoretical C_2H_2/N_2 conversion factor of 3:1 has commonly been used. However, measurements in the field

of the conversion ratio have been found to vary (Hardy *et al.* 1973; Amager *et al.* 1979; Hansen *et al.* 1987).

The ratio of C_2H_2/N_2 may vary due to soil total N and climatic conditions. Marriott (1988) reported different molar ratios in clover for C_2H_2/N_2 reduced, ranging from 2.9 to 9.5 with lower values obtained during summer months. This trend highlights the problems associated with extrapolation of C_2H_2 reduction to amounts of fixed N_2 when ARA is carried out during one season. Bergersen (1970) and Hardy *et al.* (1973) emphasised that when the assay is to be used as a quantitative estimate of N_2 fixation, the theoretical conversion factor of three should not be assumed, but in all cases the specific assay system should be calibrated with other methods of estimating N_2 fixation such as the ^{15}N dilution technique.

Major errors are also likely to occur when the conditions of the assay are not carefully matched with conditions under which N_2 fixation is taking place (Bergersen, 1970). The levels of temperature, oxygen, light and moisture must duplicate those of the sample *in situ* to obtain a valid measure of fixation (Goh *et al.* 1978). Such duplication is difficult under field conditions, but attempts should be made to reduce differences where possible.

Nodule injury has been reported to decrease ARA activity. Excised nodules are less active than nodulated roots and whole plants are even more active (Hardy *et al.* 1973). Large errors are likely to arise when cores are used for the incubation in comparison to whole plants because it is difficult to recover all nodules from cores (Danso *et al.* 1992).

Incubation time has been reported to have an influence on the ARA activity, and there is considerable variation in the maximum time reported for incubation (Edmeades, 1976; Edmeades and Goh, 1978). Goh *et al.* (1978), working with white

clover found that three hour incubation gave best estimate of symbiotic N_2 fixation relative to ^{15}N technique. One hour of incubation over-estimated, while six hours of incubation underestimated the rate of symbiotic N_2 fixation. Major difficulties are also encountered in the integration of the amounts of N_2 fixed, if C_2H_2 reduction is determined over 30 minutes to three hours and converted to a day or month basis by multiplying the value obtained proportionally.

The measured rates of symbiotic N_2 fixation could be underestimated by ARA because N_2 fixing activity below the top soil may not be accounted for, depending on the depth of the soil sampled. In most cases the acetylene reduction assay greatly under estimates the rate of N_2 fixation per plant (Peoples and Herridge, 1990; Giller and Wilson, 1991).

A fundamental assumption of ARA is that the rate of nitrogenase activity is not affected by the substitution of C_2H_2 for N_2. In many legume species there can be substantial decline in nitrogenase activity after exposure to C_2H_2 (Minchin *et al.* 1983). The extent of C_2H_2-induced decline can be influenced by plant and nodule age, plant stress, nitrate treatments and change in O_2 partial pressure. This may make quantitative comparisons of N_2 fixation based on long term C_2H_2 accumulation misleading (Witty and Minchin, 1988; Peoples and Herridge, 1990).

2.6.7. ^{15}N isotope techniques

A need for direct assay in measuring N_2 fixation in the field has led to the wide spread application of ^{15}N isotope method (Gallon and Chaplin, 1987). Three ^{15}N isotope techniques that have been used to measure BNF include the ^{15}N natural abundance method and two techniques using ^{15}N labelled fertilisers (Hauck and Bremner 1976; Peoples and Herridge, 1990).

The two methods using labelled fertiliser are the ^{15}N dilution technique and the ^{15}N isotope depleted technique. The fertilisers used in the ^{15}N isotope dilution technique have isotope abundance higher than 0.366 atom% (i.e. the ^{15}N isotope abundance of atmospheric N) while the ^{15}N isotope depleted technique use fertilisers with ^{15}N isotope concentration lower than natural abundance.

2.6.7.1. Natural abundance method

The method depends on differences in the natural ^{15}N abundance in soil compared to atmospheric N_2 (Peoples *et al.* 1989a; Hamilton *et al.* 1993). Almost all N transformations in the soil result in isotope fractionation. The net effect is often a small increase in the ^{15}N abundance of soil N compared with the atmospheric N_2 (Peoples and Herridge, 1990). Enrichment of soil occurs due to isotopic discrimination during processes such as ammonia volatilisation, denitrification and other transformation of N in the soil. As the ^{15}N isotope is heavier than ^{14}N, compounds containing ^{15}N tend to react more slowly, (especially in reactions that lead to gaseous losses in soil), thus the soil becomes slightly enriched with ^{15}N (Giller and Wilson, 1991).

The technique requires a highly sensitive and accurate mass spectrometer to determine the ^{15}N isotope accurately. In the sample preparation, contamination with ^{15}N rich material should be avoided, and uniform dry samples should be prepared to avoid variation due to tissue differences in the ^{15}N abundance (Peoples and Herridge, 1990). The accuracy of this technique will depend on levels of natural ^{15}N abundance of the soil. Low and variable ^{15}N values are unsuitable for assessing N_2 fixation (Peoples and Herridge, 1990; Hamilton *et al.* 1993).

2.6.7.2. ^{15}N isotope dilution technique

This technique is based on the atom $\%^{15}N$ difference between atmospheric N_2 and soil N. The N_2 fixing plant which are able to utilise the atmospheric N_2 contain lower %atom ^{15}N compared to those that are non-N_2-fixing, and derive all their N from the soil with relatively higher %atom ^{15}N (Peoples and Herridge, 1990; Hamilton *et al.* 1993).

This technique requires the application of equal nitrogen rates and ^{15}N enrichment of a given fertiliser, to both a suitable non-N_2-fixing reference plant and the legume, respectively (Danso, 1986; Hamilton *et al.* 1993). The difference in the concentration of ^{15}N between the N_2 fixing plant and the non-N_2-fixing plant is used to determine the proportion of N in the fixing plants that is derived from the atmosphere (Peoples and Herridge, 1990; Cookson *et al.* 1990).

This method is based on three assumptions; (a) all N in reference plant is derived from the soil, so that the $^{15}N/^{14}N$ ratio in the plant tissue is the same as that of the soil mineral N; (b) the N_2 fixer and the reference plant explore a soil pool with identical $^{15}N/^{14}N$ ratios (Hamilton *et al.* 1992); and (c) the isotope incorporated into the soil N is equally available to both plants (LaRue and Patterson, 1981; Peoples and Herridge, 1990).

The proportion of N derived from the atmosphere (%Ndfa), is calculated as:-

%Ndfa = (1 - c/a) x 100.....2.1

where:-

%Ndfa is the proportion of N derived from the atmosphere,

a and c are atom% ^{15}N excesses in reference and leguminous plant respectively. Atom% ^{15}N excess = atom% ^{15}N sample - atom% ^{15}N of reference standard which is usually atmospheric N_2 gas (atom% ^{15}N of atmospheric N_2 = 0.3663)

The amount of N_2 fixed is calculated as:

Total amount of N_2 fixed = (%Ndfa) x total N in legume.....2.2

In this method, the N derived from fixation can be distinguished from N derived from the soil or fertiliser. Thus a truly integrated value for N_2 fixation for the whole growing season can be estimated qualifying the method as the most reliable (Cookson *et al.* 1990; Viera-Vargas *et al.* 1995).

2.6.7.3. Advantages of the isotope dilution technique

Unlike the total N difference method it is not necessary to grow the reference crop without the fertiliser. This method also allows comparisons of N_2 fixing abilities of different legumes to be made simply by ranking on the $^{15}N/^{14}N$ basis and the method is yield independent (Rennie, 1985; Danso, 1986). The lower this ratio the higher the amount of N_2 fixed. In such cases reference crops may be avoided (Peoples *et al.* 1989a). In addition, information such as N fertiliser use efficiency can be obtained.

2.6.7.4. Disadvantages associated with the ^{15}N isotope dilution technique

Mismatched reference crops can produce significant under- or over-estimates of fixation (Ledgard *et al.* 1985), but these are small with high fixation values (Danso and Kumarasighe, 1990). Differences in rooting patterns and an uneven fertiliser distribution with depth are the most obvious reason for plants taking up soil N at different isotopic compositions. Errors can also result from a decrease in soil enrichment with time coupled with differences in seasonal N uptake pattern of the fixing and the non-N_2-fixing plants (Witty, 1983; Chalk, 1985; Peoples *et al.* 1989a; Hamilton *et al.* 1992). So there is need to select appropriate control plants and fertiliser treatment which lead to uniform and constant enrichment of the soil (Witty *et al.* 1988).

Symbiotic N_2 fixation has been found to be inhibited by added labelled N fertiliser (Hamilton *et al.* 1991). The solution to the problem of significant and unwanted interactions between N addition and N_2 fixation is to add as little N as practical.

CHAPTER 3

MATERIALS AND METHODS

3.1. Site and trial description

3.1.1. Site description

The study was conducted at Woodhill Forest, on the west coast of the North Island of New Zealand (latitude 36^0 40' S). The forest is located 50 km north-west of Auckland on a yellow-brown coastal sand of the Pinaki suite (Cox, 1977). Nitrogen is the only element known to limit productivity in this forest (Beets and Madgwick, 1988).

The average annual rainfall is 1328 mm, while the mean air temperature is 18.4°C and 10.4°C in January and July, respectively. Some climatic variables during the study period are shown in Table 3.1.

The field work was conducted in clearfelled, first-rotation replanted *Pinus radiata* stand at a density of 1000 stems per hectare. The trial consisted of two parts; one within an older trial designed to screen legumes with potential for growth in sand dune forests as replacement for the yellow tree lupin (Figure 3.1), and the other in an operational area sown with a mixture of legumes. The two sites were about 7.5 km apart.

Before the establishment of legumes, both sites were aerially sprayed with glyphosate herbicide (Roundup) at the rates of 6-8 litres ha^{-1}.

Table 3.1. Monthly rainfall and mean air temperatures during the study period from Henderson, River Park meteorological station[#].

Year	Month	Temperature (°C)	Rainfall (mm)
1994	July	10.1	224
	August	10.7	96
	September	12.1	228
	October	13.7	154
	November	15.8	44
	December	18.7	18
1995	January	19.7	91
	February	20.6	55
	March	18.7	195
	April	18.1	89
	May	14.1	147
	June	11.6	193
	July	10.0	240

Monthly climate data extracted from New Zealand climate digest; A report by the Institute of Water and Atmosphere Research Ltd. [#] Henderson River Park the nearest meteorological station to Woodhill forest is about 25 km away.

3.1.2. Experimental design and layout

3.1.2.1. Experiment 1

In the original trial, fourteen legumes were established from seedlings in single-species plots (3.5 x 3.5m) in May, 1991 using a completely randomised design with four replicates (Figure 3.1). Data for Experiment 1 were derived from three of these species within three replicate plots. The three species were: Maku Lotus (*Lotus pedunculatus* Cav. "Grasslands Maku"), hairy canary clover (*Dorycnium hirsutum* (L) Ser.) and everlasting pea (*Lathyrus latifolius* L). Seedlings were raised at the Forest Research Institute, Rotorua, using Woodhill sand and were treated with inoculum cultures

supplied by New Zealand Department of Scientific and Industrial Research (DSIR), Grasslands and Plant Protection Divisions, before being planted out in the field at 0.5m x 0.5m spacing.

Lat [1] **lat.**	Tri. sub.	Tri. amb.	Vic. vil.	Mel. ind.	Dor. hir.	Mel. ind.
Vic. vil.	Mel. ind.	Ast. cic.	**Lat. lat.**	Tri. amb.	Lat. lat.	
Lot. ped.	Mel. alb.	Vic. vil.	Tri. sub.	**control**	Lup. pol.	
Tri. dub.	Cor. var.	**Dor. hir.**	Mel. ind.	**Dor. hir.**	Tri. dub.	**control**
Tri. sub.	Lot. ten.	**Lat. lat.**	Mel. alb.	Lup. pol.	Vic. vil.	Mel. ind.
Dor. hir.	**control**	Lot. cor.	Cor. var.	**Lot. ped.**	Tri. sub.	
Lup. pol.	Tri. amb.	Tri. dub.	Lot. ten.	Tri. dub.	Lot. ten.	
Mel. alb.	Lot. cor.	Lup. pol.	**Lot. ped.**	Lot. cor.	Mel. alb.	Lot. ped.

Figure 3.1. Plot layout for experiment 1 consisting of *Lotus, Dorycnium, Lathyrus* and a non-legume (control) plots. [1]Only plots in bold were considered for the present study. Full names of the species are shown in Appendix 1.

3.1.2.2. Experiment 2

In this field, 3 kg ha^{-1} of Maku lotus and 10 kg ha^{-1} of serradella *(Ornithopus sativus* Brot. "Grasslands koha") were oversown in April, 1992 as a pasture sward. Ammonium superphosphate, boron (borate) and sulphur (Durasil) fertilisers were broadcast in May, 1992 at rates of 209, 1 and 40 kg ha^{-1}, respectively. A third legume, volunteer white clover *(Trifolium repens)* was widespread in the area. Four randomly-located plots were marked out in this area.

3.1.2.3. Plot layout

In both experimental sites, the study was conducted for twelve months, between July, 1994 and July, 1995. A 3m x 3m area was used in each of the legume plots. Within this area, a 1 m^2 ^{15}N treatment plot (TP) was demarcated. The surrounding area was designated the plot surround (PS), (Figure 3.2).

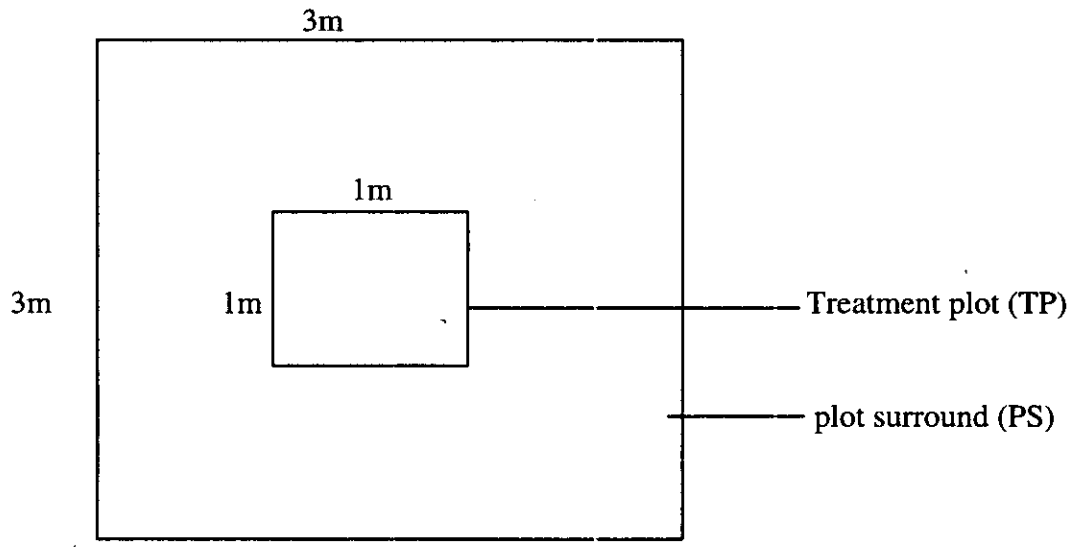

Figure 3.2. Layout of the experimental plots consisting of ^{15}N treatment plot (TP) at the centre, and the plot surround (PS).

3.2. Nitrogen-15 isotope dilution technique procedures

3.2.1. Fertiliser preparation and application procedures

3.2.1.1. Labelled fertiliser procedures

A 30% atom ^{15}N-enriched ammonium sulphate ($(NH_4)_2SO_4$) solution was prepared from a 60.4% ^{15}N-enriched $(NH_4)_2SO_4$ source. A mixture of 5.67 g unlabelled $(NH_4)_2SO_4$ and 5.52 g of 60.4% ^{15}N-enriched $(NH_4)_2SO_4$ source was diluted in 130 ml deionised water to make a 30% atom ^{15}N enriched $(NH_4)_2SO_4$ stock solution. A 10 ml aliquot of this stock solution, containing 182.5 mg of N, was diluted in 1 litre of water and applied to the TP area. Care was taken in mixing and in sprinkling it evenly on the ^{15}N treatment plot. A further 3 litres of water was sprinkled to wash the fertiliser off the plants into the soil. The ^{15}N labelled fertiliser was applied in July and November, 1994 and in February, 1995. The total N applied in these three applications was 5.547 kg N ha^{-1} (Calculation shown in Appendix 2).

3.2.1.2. Unlabelled fertiliser procedures

On each occasion of labelled fertiliser application, the PS was treated with unlabelled N at the same rate of N as the ^{15}N applied to the TP area. A 260 ml stock solution of unlabelled fertiliser containing 89.5 g $(NH_4)_2SO_4$ was prepared (Calculation shown in Appendix 3). A 20 ml aliquot of this solution was mixed with 8 litres of water and applied to the PS areas. This was followed with 24 litres of water.

3.3. Above-ground biomass sampling from the plot surround (PS)

Above-ground live biomass was harvested at ground level with hand-held shears, in July and November, 1994 and February, 1995 from a 0.5m x 0.5m area within the PS area. At each sampling date a different area was demarcated. This biomass was separated into target legume and other species. *Dorycnium* was further separated into leaf and stem components. This material was then rinsed with water and oven dried at 60 °C to a constant weight.

3.4. Total N and ^{15}N atom % determinations

In November, 1994 and February 1995, above-ground "grab" samples (obtained by randomly cutting 4-5 sub-samples from the TP and bulking them), were harvested prior to the ^{15}N fertiliser application. These samples were separated into target legume and reference grass. Yorkshire fog (*Holcus lanatus)* was used as the reference grass in November, 1994 and July, 1995 while sweet vernal grass (*Anthoxanthum odoratum)* was used as reference in February, 1995 as yorkshire fog was not sufficient during this period. Initial analysis of the two grasses showed that there were no significant differences in their ^{15}N levels, proportion of nitrogen derived from the atmosphere (%Ndfa) and N_2 fixed, estimated by using the two grasses (Appendix 4). The plant material was rinsed with distilled water to remove any attached soil and dust particles, then oven dried for 3 days at 60°C. The "grab" samples were ground through a Cyclotec 1092 sample mill. The ground material was then analysed for total N and atom% ^{15}N using a commercial continuous flow C-N analyser connected to an isotopic ratio mass spectrometer (ANCA-MS, Europa UK) (Goh *et al.* 1995). The proportion of nitrogen derived from the atmosphere (%Ndfa) and total N_2 fixed were calculated according to equations 2.1 and 2.2 (Section 2.6.7.2).

3.5. Biomass and Nitrogen accumulation in the ecosystem

Ecosystem as used in this study refers to the total above-ground dry matter, (live and dead as well as litter), below-ground (root) biomass, and the top 80 cm of soil. In this work litter was grouped with the above-ground dead dry matter since it was difficult to separate the two.

3.5.1. Final biomass harvesting in July 1995

At the end of the experiments, in July, 1995, total above-ground plant material was harvested at ground level from a 0.5m x 0.5m plot in the ^{15}N treated (TP) area (Figure 3.2). This material was separated into live legume biomass, reference grass, other plant species and dead component. On this occasion, roots were also harvested from each TP. Root sampling was done to 40 cm soil depth at 10 cm depth intervals. Soil was excavated from 30 cm x 30 cm area and passed through a 5 mm sieve to collect the roots. Root samples from the 0-10 cm soil depth were wet sieved to remove the soil and debris. It was difficult to separate roots according to species, and so all roots from each depth interval in a plot were bulked as one sample.

For this study, it was necessary to have non-legume control plots. Three control plots measuring 0.5m x 0.5m were demarcated in areas where legumes had failed during the first year of establishment or areas where none had been planted (Figure 3.1). The main species in the control plots included yorkshire fog (*Holcus lanatus*) and sweet vernal grass (*Anthoxanthum odoratum*). No legume or bare ground were present. Total above-ground plant material was harvested at ground level from this area and separated into live and dead components. Roots were also harvested following the same procedures as in the legume plots.

All the above- and below-ground dry matter samples were rinsed with water and oven dried at 60°C to a constant weight. The dried material was subsampled for N concentration and %atom ^{15}N analysis following procedures described in Section 3.4.

3.5.2. Soil sampling procedures

Soil samples were collected from 5 depths in the TP areas in July, 1995 at 0-10, 10-20, 20-30, 30-40 and 40-80 cm soil depths. From each TP area, two cores from each depth were bulked. Soil samples were then air dried, sieved through a 2 mm sieve and ground by use of a grinder (N.V. Temma type T250) for 1 minute. Soil total N was determined following procedures described in Section 3.4.

Bulk density soil cores, 10 cm long and 4.7 cm diameter were sampled to 40 cm depth and one core bulked from 40-80 cm depth. From each TP, one core was collected per depth interval. These soil samples were oven dried to a constant weight at 70°C and the weight expressed in g weight of soil m^{-2}.

Total amount of N in above- and below-ground plant dry matter, and in the bulk density soil samples was calculated and expressed in g N m^{-2}.

3.6. Acetylene Reduction Assay (ARA)

3.6.1. Field sampling and incubation

Acetylene for the assay was produced on the same day of use by adding water to commercial carbide (CaC_3), and collecting the generated gas in an inflatable beach ball. Immediately after the plant tops were severed for biomass determination in November 1994, February and July, 1995, legume root material was collected from each plot using a metal core sampler (10.8 cm diameter x 8.5 cm deep). The core sampler was centred on individual root system except for *Lotus* which had formed a dense

network of rooting stolons. This was to ensure that the majority of the root system of the harvested plant were incorporated. The corer was then dug out using a spade. For *Dorycnium* and *Lathyrus*, the tap root was cut parallel to the bottom of the corer using hand-held shears. The cored material was then placed on a board and vertically cut into two equal portions.

One of these portions was then placed in a jar (1040 ml). The jar was covered with a lid containing a rubber seal. By inserting a syringe through the rubber seal, 100 ml of air was removed before 100 ml of acetylene was added. This gave a concentration of 9.6% acetylene in the jar. The syringe was pumped several times to ensure thorough mixing of acetylene and air. Blank gas samples, prepared by mixing acetylene gas with air in a jar without soil or root material, were subjected to the same treatment as the previous gas samples. These samples were prepared to correct for the background ethylene concentration. After 30 minutes incubation at ambient temperature in the shade, a syringe was inserted through the rubber seal and pumped several times to mix the gases. Duplicate 10 ml gas samples were withdrawn from the jar using syringes. The gas samples were retained in the syringes by inserting the needle tips into a rubber bang, before being transported to the Forest Research Institute laboratory, Rotorua for ethylene analysis. The amount of ethylene produced after the incubation period was determined by gas chromatography.

3.6.2. Laboratory analysis

Gas samples (1 ml) were analysed on a gas chromatography system fitted with a flame ionisation detector with a glass column (5' x 1/4"), packed with poropak N (<100 mesh) and maintained at 95°C. Nitrogen was used as the carrier gas. Ethylene production was measured using acetylene peak as an internal standard, with reference to

calibration standards. (Calculation of moles of ethylene production is shown in Appendix 5). The moles of ethylene produced after 30 minutes incubation were converted to moles of N_2 fixed using the theoretical molar ratio of 3:1. To obtain a valid comparison between the ^{15}N dilution technique and the ARA, results from the later were calculated in terms of N_2 fixed m^{-2} of soil surface in 122 days, the time interval used in the estimation of N_2 fixation by ^{15}N method.

3.7. Statistical analysis

Statistical analysis of data was computed using Statistical Analysis System (SAS) (SAS Institute Inc. 1990). Comparisons among treatments means were made on the basis of least significant difference (LSD) at 5% probability. For some parameters log-transformation was conducted prior to data analysis; in such cases the logarithmically transformed means are reported. Stepwise regression analysis was also used to examine the relationships between the N_2 fixed and dry matter yield increments and N concentration in the above-ground material of the legume. For experiment 2, some comparisons between the legume and non-legume (control) plots were performed by linear contrast.

CHAPTER 4

RESULTS

4.1. Experiment 1: Dry matter yield and biological N_2 fixation

4.1.1. Above-ground dry matter yield

4.1.1.1. Seasonal biomass yield

Seasonal total above-ground biomass yield, which included legume and other plant species showed a similar growth trend in *Lotus* and *Lathyrus* plots (Table 4.1). Above-ground biomass from these plots was highest in spring (November) and lowest in summer (February) with winter (July) being intermediate. However, *Dorycnium* plot showed no significant seasonal changes in total biomass although the dry matter in summer and winter were 41 and 26% less than that in spring respectively.

In comparing the treatments, *Dorycnium* plot showed the highest biomass in summer and winter, while in spring its biomass did not differ significantly from that of *Lathyrus*.

4.1.1.2. Proportion of legume in total above-ground biomass

At all sampling dates, legumes constituted between 58 and 93% of the total biomass (Table 4.2). However, there were no seasonal differences in the proportion of legume within a species. Species differences were only observed in winter (July), when the proportion of legume in *Lotus* plot was significantly lower than that in *Dorycnium*.

The annual mean legume proportion ranged between 60 and 87%, with *Dorycnium* showing a significantly higher percentage of legume than *Lotus*.

Table 4.1. Total above-ground biomass from *Lotus*, *Dorycnium* and *Lathyrus* plots in spring (November 1994), summer (February 1995) and winter (July 1995).

Plot	Biomass yield ($g\ m^{-2}$)		
	Spring	Summer	Winter
Lotus	252bA	33bB	131cA
Dorycnium	828aA	490aA	609aA
Lathyrus	573aA	93bC	229bB
CV (%)	4.7	16.5	4.5

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between plots and differences between seasons for each plot, respectively. Values are back transformed means.

Table 4.2. Proportion of legume in total above-ground biomass in spring (November 1994), summer (February 1995) and winter (July 1995).

Species	Proportion of legume (%)			Annual
	Spring	Summer	Winter	mean (%)
Lotus	58aA	62aA	60bA	60b
Dorycnium	83aA	86aA	93aA	87a
Lathyrus	77aA	73aA	88abA	80ab
CV (%)	25.3	23.0	19.0	16.0

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively.

4.1.1.3. Legume dry matter yield

In comparing the legumes, the highest total dry matter yield (DMY) was shown by *Dorycnium* in summer (February) and winter (July) (Table 4.3). However, in spring (November), the DMY of *Dorycnium* did not differ significantly from that of *Lathyrus,* while in summer, there was no significant difference between *Lotus* and *Lathyrus* DMY.

Dry matter yield of *Dorycnium* did not differ significantly between seasons, while significantly lower DMY was produced by *Lotus* and *Lathyrus* in summer (February), being approximately 85% less than that produced in spring (November).

For non-woody components, all legumes showed similar seasonal growth patterns (Figure 4.1). For each legume biomass was higher in spring (November), decreased sharply in summer (February), and then increased gradually in winter (July). *Dorycnium* woody component showed a different growth pattern, with continuous biomass yield up until summer (February) before it decreased in winter (July) (Figure 4.1). This component formed 56, 89 and 76% of *Dorycnium* DMY in spring, summer and winter, respectively.

Table 4.3. Above-ground legume dry matter yield (DMY) in spring (November 1994), summer (February 1995) and winter (July 1995).

Species	Legume DMY ($g\ m^{-2}$)		
	Spring	Summer	Winter
Lotus	141.2bA	20.1bB	73.8cA
Dorycnium	678.6aA	419.9aA	562.7aA
Lathyrus	432.7aA	66.7bB	201.4bA
CV(%)	8.2	16.6	6.5

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively. Values are back transformed means.

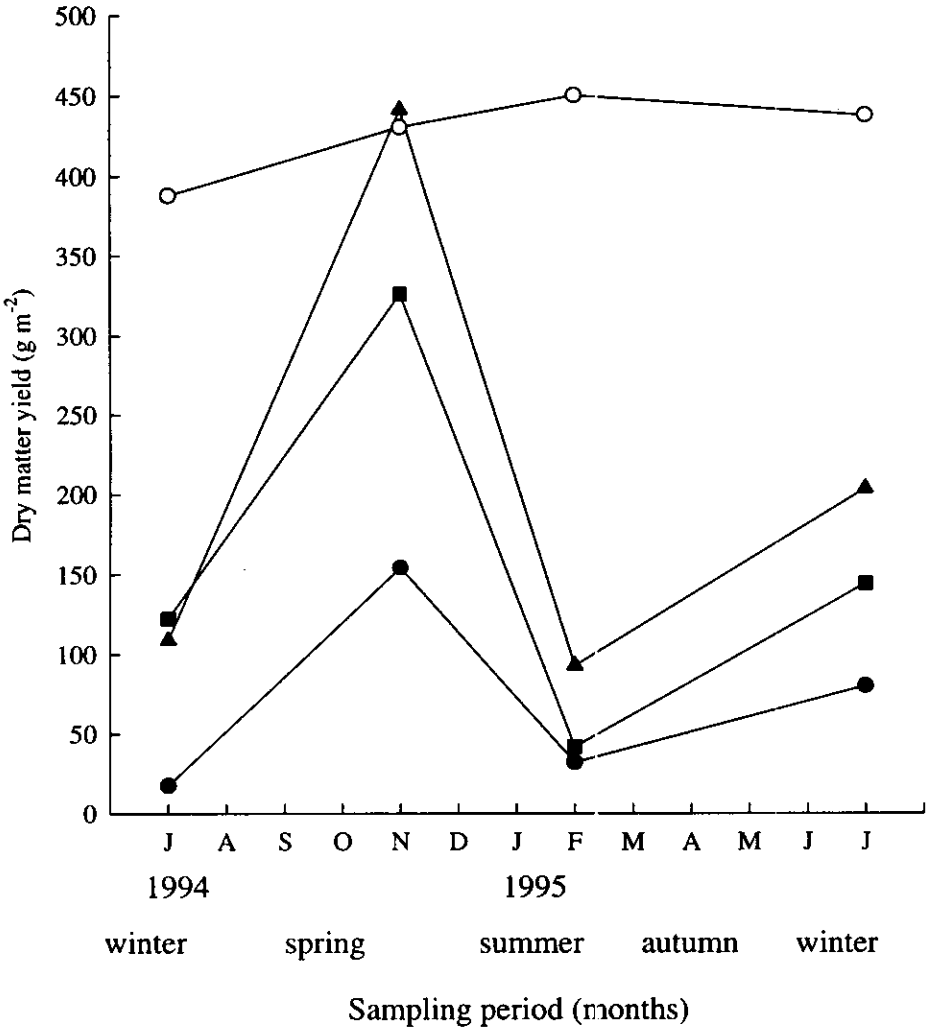

Figure 4.1. Seasonal non-woody biomass of *Lotus* (●), *Dorycnium* (■) and *Lathyrus* (▲) and woody dry matter yield of *Dorycnium* (○).

4.1.1.4. Legume dry matter yield increment

All the legumes showed a similar pattern of dry matter increment (Figure 4.2). Generally, increments were highest between winter and spring (July-November) constituting 61, 59 and 62% of the annual increment for *Lotus*, *Dorycnium* and *Lathyrus*, respectively. During this period *Lathyrus* dry matter increment was 63 and 41% more than that of *Lotus* and *Dorycnium*, respectively.

While *Lathyrus* showed a significantly higher yield increment between winter and spring (July-November) than in the other seasons, *Dorycnium* showed no seasonal differences (Figure 4.2). The annual DMY increment for *Lathyrus* was 1.5 and 2.7 times those of *Dorycnium* and *Lotus*, respectively.

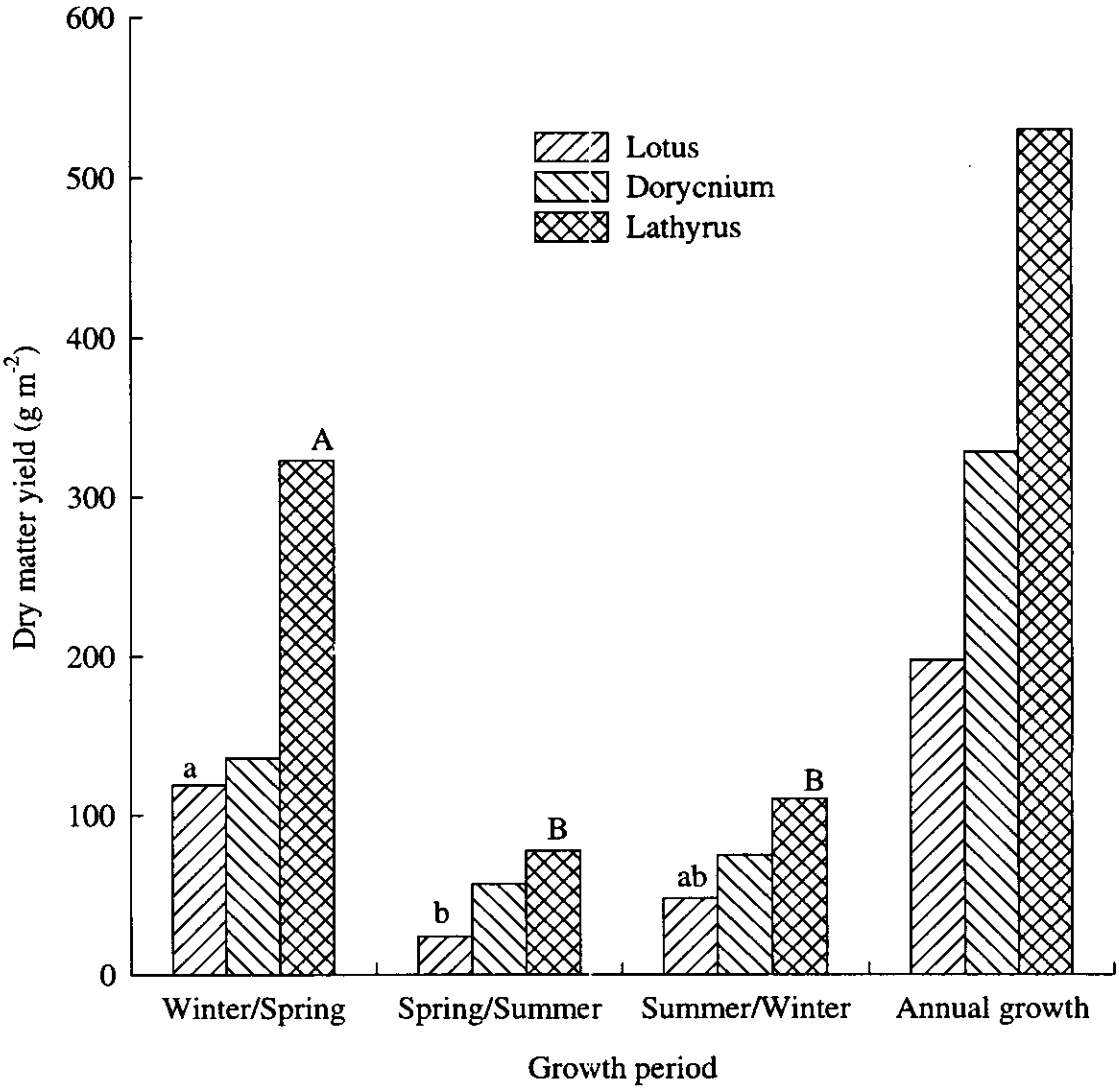

Figure 4.2. Seasonal and annual dry matter yield increment of *Lotus*, *Dorycnium* and *Lathyrus* between winter 1994 and 1995. Only significant results are indicated. Small and capital letters indicate seasonal differences for *Lotus* and *Lathyrus*, respectively.

4.1.2. Above- and below-ground plant dry matter accumulation

4.1.2.1. Live and dead above-ground dry matter accumulation

In July, 1995 no differences were observed in the live and dead dry matter components from *Lotus* and *Lathyrus* plots, while for the *Dorycnium* and the control plots significant differences were observed (Table 4.4). Non-living material constituted 65% and 64% of the total dry matter in *Dorycnium* and the control plots, respectively. *Dorycnium* plot showed the highest above-ground total dry matter accumulation while those of *Lotus* and control, which did not differ from each other, showed the lowest (Table 4.4).

Table 4.4. Above-ground live and dead dry matter accumulation in legume and control plots in July 1995, after four years of legume growth.

Component	Dry matter ($g\ m^{-2}$)				
	Lotus	Dorycnium	Lathyrus	Control	CV (%)
Live	131aC	609bA	229aB	140bC	4.2
Dead	190aC	1133aA	328aB	247aBC	4.0
Total	321C	1742A	557B	387C	2.2

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between live and dead dry matter components and differences between treatments, respectively. Values are back transformed means.

4.1.2.2. Root dry matter yield (RDMY), July 1995

Generally, RDMY decreased with an increase in soil depth (Figure 4.3). *Lathyrus* plot showed a gradual decline between 0-10 and 20-30 cm depths, while the other plots showed a sharp decline between 0-10 cm to 10-20 cm depth. At the 0-10 cm soil depth, *Dorycnium* treatment showed the highest RDMY which was 42, 63 and 70% more than that from *Lotus*, *Lathyrus* and the control plots, respectively. At 0-10 cm depth, root dry matter from *Dorycnium* plot was not significantly different from that of *Lotus* (Table 4.5). Below 10 cm depth, the control plot showed significantly low root dry matter yield (Table 4.5).

Table 4.5. Log-transformed values for root dry matter from legume and control plots in July 1995.

Soil depth (cm)	Log root DMY					
	Lotus	Dorycnium	Lathyrus	Control	CV (%)	SE
0-10	5.9AB	6.5A	5.4B	5.2B	6.4	0.37
10-20	4.4A	4.8A	5.2A	3.1B	10.0	0.43
20-30	3.3B	4.2AB	4.8A	2.0C	18.4	0.66
30-40	3.0A	4.1A	3.4A	1.4B	27.1	0.81

Values followed by the same letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between treatments.

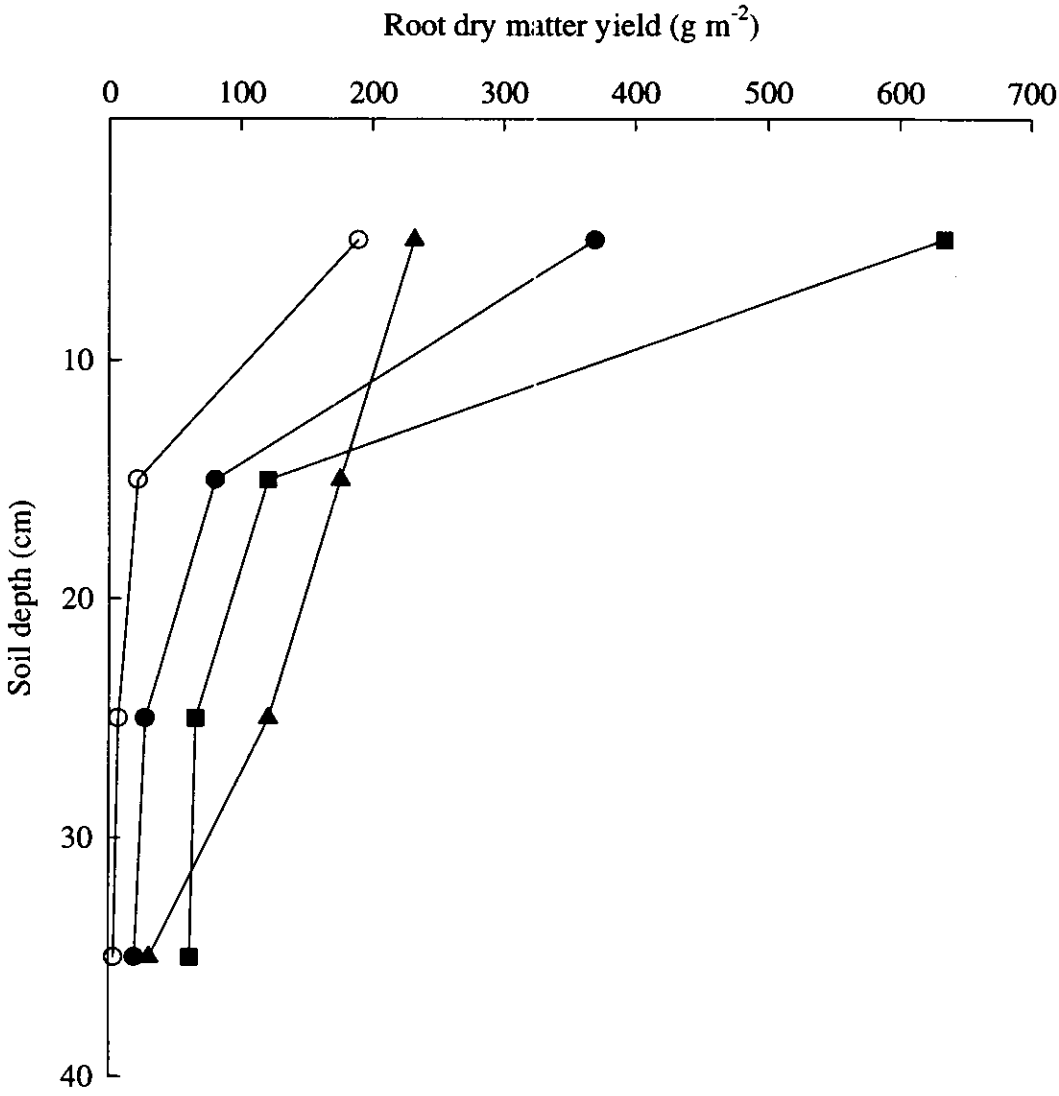

Figure 4.3. Root dry matter yield from *Lotus* (●), *Dorycnium* (■), *Lathyrus* (▲) and control (○) plots in July 1995.

4.1.2.3. Above- and below-ground dry matter distribution

In *Lotus* and *Lathyrus* plots, dry matter appeared to be fairly equally distributed above- and below-ground (Table 4.6). However, in *Dorycnium* and the control plots, significantly more material accumulated above ground, constituting 67% and 63% of total dry matter. The total amount of root biomass accumulated by *Dorycnium* did not differ significantly from that of *Lathyrus* (Table 4.6) although *Dorycnium* had 33% more biomass. However, *Dorycnium* accumulated significantly more dry matter after four years of growth.

Table 4.6. Comparison of above- and below-ground dry matter accumulation in the legume and control plots in July 1995, after four years of legume growth.

Component	Dry matter ($g\ m^{-2}$)				
	Lotus	Dorycnium	Lathyrus	Control	CV (%)
Above-ground	321aC	1742aA	557aB	387aC	2.2
Below-ground	463.6aB	876.5bA	589aAB	226.1bC	3.9
Total	784.6C	2618.5A	1146B	613.1C	2.1

Values followed by the same small in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between above- and below-ground dry matter components and differences between treatments, respectively. Values are back transformed means.

4.1.3. Nitrogen concentration and yield in dry matter

4.1.3.1. Nitrogen concentration in legume above-ground live component

Lathyrus showed the highest nitrogen (N) concentration in spring (November) and summer (February), while in winter (July) its N concentration was not significantly different from that of *Lotus* (Table 4.7). *Dorycnium* showed the lowest N concentration at all sampling dates. Generally, in absolute values, there was an increase in N concentration from spring (November) to summer (February). However, this increase was not significant for *Dorycnium.* While this concentration was maintained for *Dorycnium* and *Lotus,* it was decreased by 20% from summer to winter in *Lathyrus.*

The annual mean N concentration was highest for *Lathyrus,* while that of *Dorycnium* was not significantly different from that of *Lotus* even though there was a 26% difference in their N concentrations (Table 4.7).

Table 4.7. Nitrogen concentration in the above-ground legume biomass in spring (November 1994), summer (February 1995) and winter (July 1995).

Species	Nitrogen concentration (%)			Annual
	Spring	Summer	Winter	mean
Lotus	3.0bB	3.6bA	3.6aA	3.4b
Dorycnium	2.4cB	2.5cAB	2.7bA	2.5b
Lathyrus	4.3aB	5.0aA	4.0aC	4.4a
CV%	3.5	8.2	4.9	10.6

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively.

4.1.3.2. Seasonal nitrogen yield in the above-ground legume biomass

Seasonal and annual N yields differed among legume species. In comparing the species, *Lathyrus* produced significantly higher N yield between winter and spring (July-November) than *Dorycnium* and *Lotus* which had similar N yield during this period (Table 4.8). However, while N yield did not differ significantly among legume species between summer and winter (February-July), only *Lotus* showed significantly lower N yield than *Lathyrus* between spring and summer (November-February) period. For individual species, *Lotus* showed no significant differences in seasonal N yield (Table 4.8), while *Lathyrus* showed significantly higher N yield between winter and spring.

Lathyrus produced the highest annual N yield which was three and three and half times higher than that of *Dorycnium* and *Lotus,* respectively. However, there were no significant differences between *Dorycnium* and *Lotus* N yields.

Table 4.8. Seasonal and annual nitrogen yield of above-ground legume biomass between July 1994 and July 1995.

Species	Legume biomass N yield (g m^{-2})			Annual
	Winter/Spring	Spring/Summer	Summer/Winter	N yield
Lotus	3.5bA	0.61bA	1.7aA	6.2b
Dorycnium	4.0bA	1.2abB	2.0aAB	7.3b
Lathyrus	13.9aA	3.9aB	4.3aB	22.2a
CV%	32.0	37.1	30.7	20.4

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively. Values are back transformed means.

4.1.3.3. Nitrogen accumulation in live and dead above-ground plant dry matter components by July 1995

Only *Lathyrus* showed significant differences between the N yield in the live and dead dry matter components, with the live biomass component yielding 67% of the total N (Table 4.9). *Lathyrus* and *Dorycnium* plots showed higher N yield in their live biomass than *Lotus* and control, which did not differ from each other. However, *Dorycnium* plot showed the highest N accumulation in its dead dry matter component and the highest total N yield, while *Lotus* and control showed the lowest N accumulation in these components (Table 4.9).

Table 4.9. Nitrogen accumulation in above-ground live and dead dry matter from legume and control plots in July 1995, after four years of legume growth.

Component	Nitrogen accumulation (g m^{-2})				
	Lotus plot	Dorycnium plot	Lathyrus plot	Control plot	CV%
Live	4.0aB	8.7aA	9.2aA	2.5aB	16.5
Dead	2.4aC	10.3aA	4.6bB	1.8aC	23.5
Total	6.4C	19.0A	13.8B	4.3C	9.7

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between live and dead plant dry matter components and differences between species, respectively. Values are back transformed means.

4.1.3.4. Nitrogen yield in roots

Root N yield decreased with increased soil depth for all plots (Figure 4.4). This decrease was gradual for *Lathyrus* from 0-10 to 20-30 cm depths. The other plots however, showed sharp decline in N yield between 0-10 cm to 10-20 cm depth.

Nitrogen yield in *Dorycnium* plot was about 35% more than that in *Lotus* and *Lathyrus* plots in the 0-10 cm soil depth. Control plot showed the lowest N yield at all depths but was not significantly different from that of *Lotus* plot between 10-20 and 20-30 cm depths (Table 4.10).

Table 4.10. Log-transformed values for nitrogen yield in root in July 1995.

Soil depth (cm)	Log N yield in roots					
	Lotus	Dorycnium	Lathyrus	Control	CV (%)	SE
0-10	1.7A	2.2A	1.7A	0.9B	23.6	0.38
10-20	-0.3BC	0.5AB	1.5A	-1.3C	65.0	0.67
20-30	0-1.8BC	-0.3AB	1.1A	-2.6C	102.0	0.91
30-40	-1.9B	-0.2A	-0.2A	-4.3C	50.3	0.84

Values followed by the same letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between treatments.

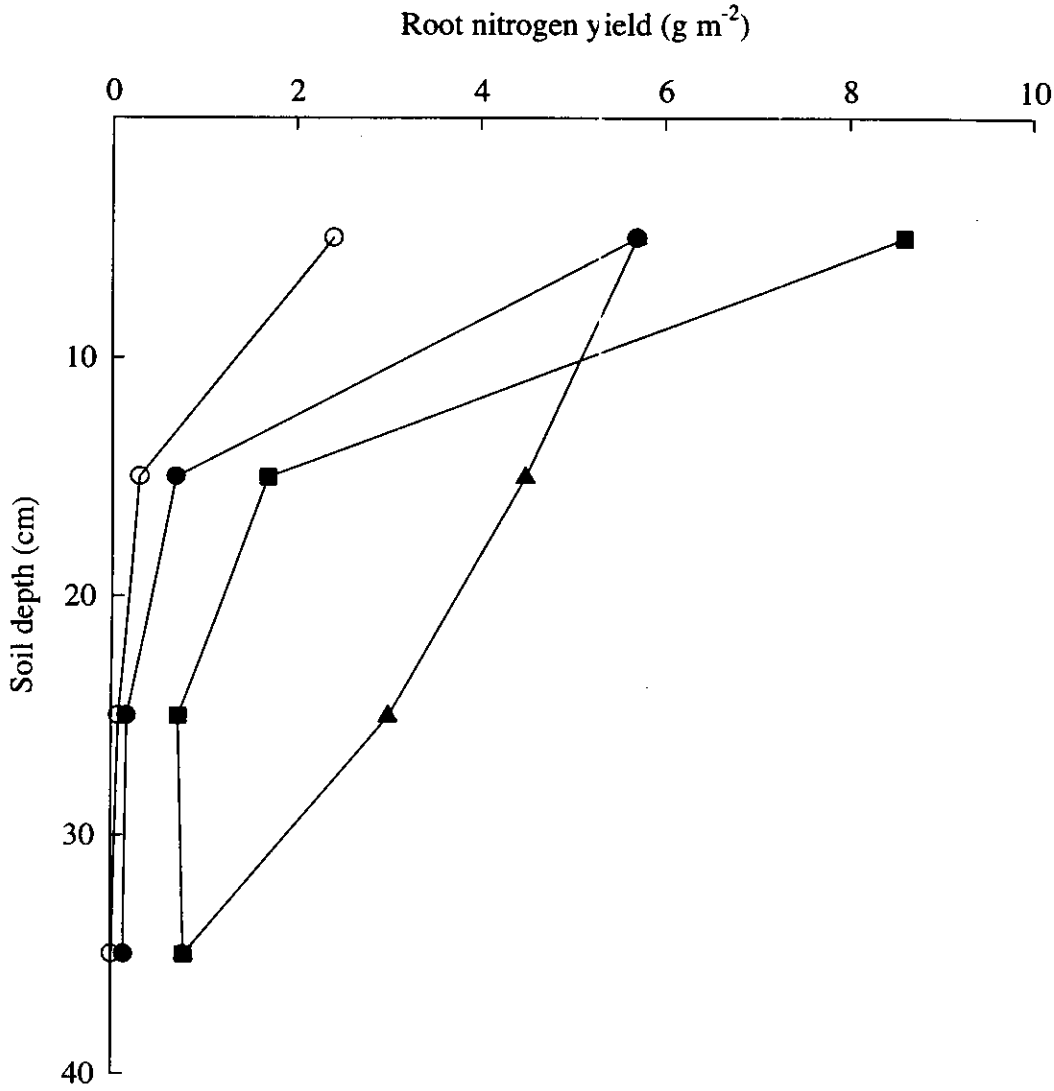

Figure 4.4. Root nitrogen yield in *Lotus* (●), *Dorycnium* (■), *Lathyrus* (▲) and control (○) plots in July 1995.

4.1.4. Total soil N at different depths

The amount of total soil N decreased with an increase in soil depth (Figure 4.5). All the plots showed a sharp decline in N between 0-10 and 10-20 cm depths, below which there was a gradual decrease. No significant treatment differences were observed in the amount of soil N at each depth (Table 4.11).

Table 4.11. Log-transformed values for total soil nitrogen in July 1995.

Soil depth (cm)	Log total soil N						
	Lotus	Dorycnium	Lathyrus	Control	CV (%)	Sig.*	SE
0-10	4.4	4.7	4.7	5.0	9.8	NS	0.46
10-20	3.5	3.0	3.4	3.2	18.2	NS	0.60
20-30	3.4	2.7	2.9	3.1	31.4	NS	0.94
30-40	2.2	2.5	2.7	2.9	36.8	NS	0.94
40-50	2.1	2.2	2.0	1.8	48.0	NS	0.98

Sig.* = Significance
NS = Not significant at LSD ($P \leq 0.05$)
Soils were dried at 70°C

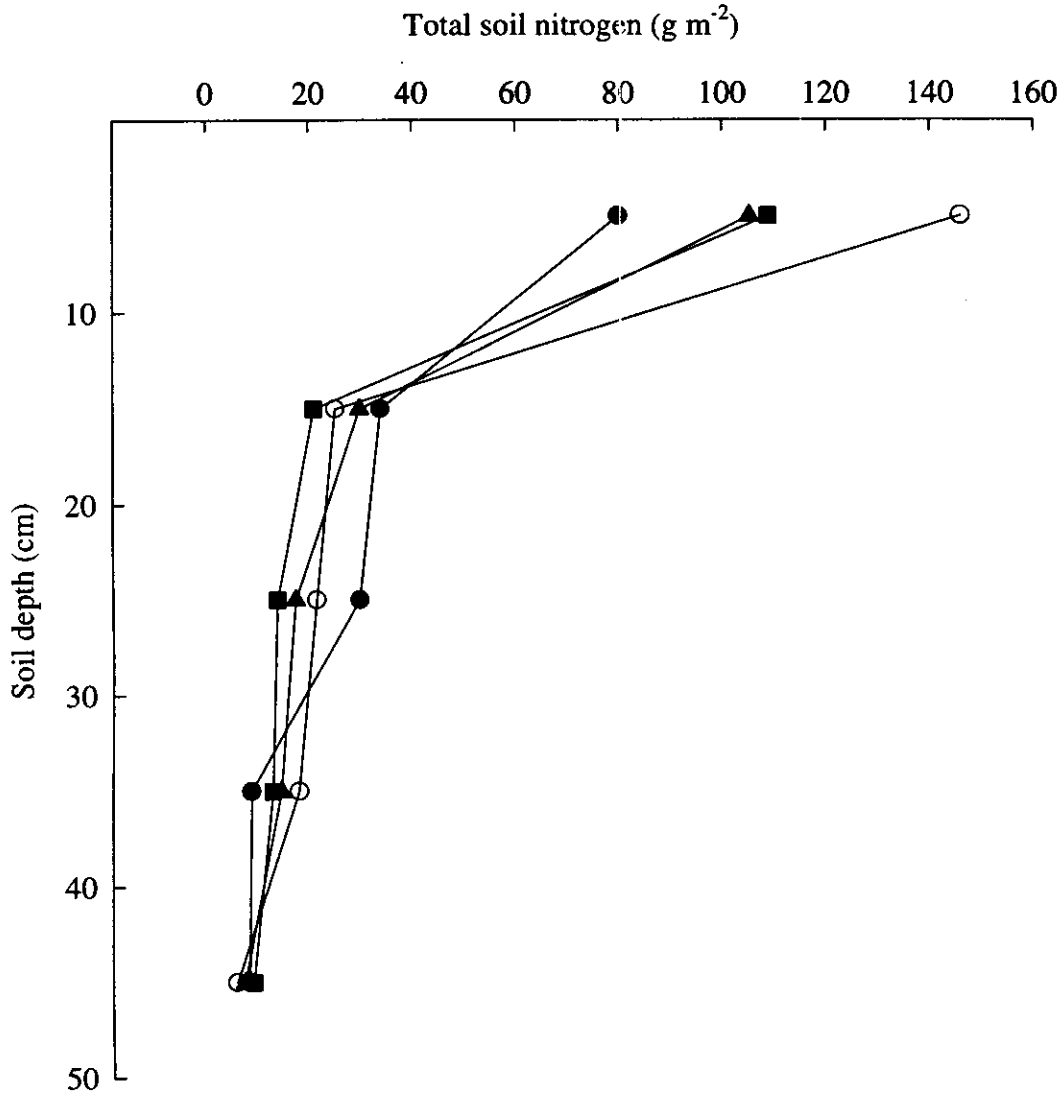

Figure 4.5. Total soil nitrogen at different depths for *Lotus* (●), *Dorycnium* (■), *Lathyrus* (▲) and control (○) plots in July 1995.

4.1.5. Total amount of nitrogen in the ecosystem in July 1995

Nitrogen yield in above-ground dry matter from *Dorycnium* and control plots was significantly higher than that from the below-ground dry matter. This constituted 62% of their individual total dry matter N, while that of *Lotus* and *Lathyrus* constituted about 46% (Table 4.12). Total dry matter N yield was highest in *Dorycnium* and *Lathyrus* plots, intermediate in *Lotus,* while control plot showed the lowest. However, total soil N and the ecosystem N did not differ significantly between treatments.

Table 4.12 Nitrogen distribution in above- and below-ground dry matter and in the top 80 cm soil depth in July 1995, after four years of legume growth.

Component	Total N ($g\ m^{-2}$)				
	Lotus plot	Dorycnium plot	Lathyrus plot	Control plot	CV (%)
Above-ground DM	6.4aC	19.0aA	13.8aB	4.3aC	9.7
Below-ground DM	7.2aB	12.0bA	16.0aA	2.6bC	12.0
Total N in DM	13.8B	31.0A	29.8A	6.9C	5.7
Soil total N (0-80cm)*	179.0A	171.4A	186.3A	235.9A	8.1
Total ecosystem N (above and below)	230.0A	241.5A	250.3A	261.6A	6.7

Values followed by the same small letter in a column or capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between above- and below-ground components and differences between treatments for each component, respectively. Values are back transformed means.

*Soils were dried at 70°C

4.1.6. Biological nitrogen fixation

4.1.6.1. Proportion of nitrogen derived from the atmosphere (%Ndfa)

Significant differences in the %Ndfa among legume species were observed between spring and summer (November-February) and summer and winter (February-July), when *Lotus* derived the lowest amount of N_2 from the atmosphere (Table 4.13). Seasonal differences in the %Ndfa for each legume were only observed in *Lotus* which derived a significantly higher amount of N from the atmosphere between winter and spring in comparison to the period between spring and summer. On average the annual mean %Ndfa of *Dorycnium* and *Lathyrus* was about 98 and 95%, respectively, and was significantly higher than that of *Lotus* (Table 4.13).

Table 4.13. Seasonal and annual proportion of nitrogen derived from the atmosphere (%Ndfa) in the above-ground legume biomass between July 1994 and July 1995.

Species	%Ndfa			mean
	Winter/Spring	Spring/Summer	Summer/Winter	annual
Lotus	96.8aA	67.1bB	78.1bAB	80.7b
Dorycnium	98.5aA	98.1aA	96.7aA	97.8a
Lathyrus	97.3aA	95.2aA	92.8aA	95.1a
CV%	2.1	15.8	7.0	9.6

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively.

4.1.6.2. Amount of nitrogen fixed in above-ground legume biomass

Between winter and spring (July-November), *Lathyrus* fixed the highest amount of N (Table 4.14). However, in the other seasons, the amount of N_2 fixed by *Lathyrus* did not differ significantly from that fixed by *Dorycnium*. Amount of N_2 fixed by *Lotus*, *Dorycnium* and *Lathyrus* between winter and spring (July-November) constitued 62, 55 and 64% of their annual fixation, respectively, while between spring and summer (November-February), these species fixed only 9.6 and 17 and 17% of the total annual amount, respectively.

Lathyrus fixed the highest amount of N (21.4 g N m^{-2} yr^{-1}), which was three to four times that fixed by *Dorycnium* and *Lotus*, respectively. Even though *Dorycnium* fixed 22% more N than *Lotus*, these two species did not differ significantly in their annual N_2 fixation (Table 4.14).

Table 4.14. Seasonal and annual amounts of nitrogen fixed in above-ground legume biomass between July 1994 and July 1995.

Species	Total N (g m^{-2})			Total N_2
	Winter/Spring	Spring/Summer	Summer/Winter	fixed yr^{-1}
Lotus	3.4bA	0.53bB	1.3bAB	5.5b
Dorycnium	3.9bA	1.2abB	1.9abAB	7.1b
Lathyrus	13.6aA	3.7aB	4.0aB	21.4a
CV%	22.3	41.8	31.1	20.5

Values followed by the same small letter in a column and capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between legumes and differences between seasons for each legume, respectively. Values are back transformed means.

4.2. Experiment 2: Biomass yield and biological N_2 fixation in the mixed legume sward

4.2.1. Biomass yield

4.2.1.1. Seasonal biomass yield

Total biomass, legume DMY and legume DM increment showed similar seasonal patterns with highest values in spring (November) and lowest in summer (February) (Figure 4.6). Legume growth increment in spring constituted 70% of the annual production. The proportion of legume in the total above-ground biomass was lowest in summer (February) constituting about 43% of the total biomass, while in spring (November) and winter (July) it constituted about 82% of the total biomass.

4.2.1.2. Above- and below-ground plant dry matter accumulation

4.2.1.2.1. Above-ground live and dead dry matter accumulation

Even though above-ground live biomass component constituted 57% of total dry matter in the legume plot, this amount was not significantly different from that of the dead dry matter component (Table 4.15). In constrast, the control plot accumulated significantly more dry matter in its dead component. Total dry matter accumulation in the legume plot was 34% higher than that of the control.

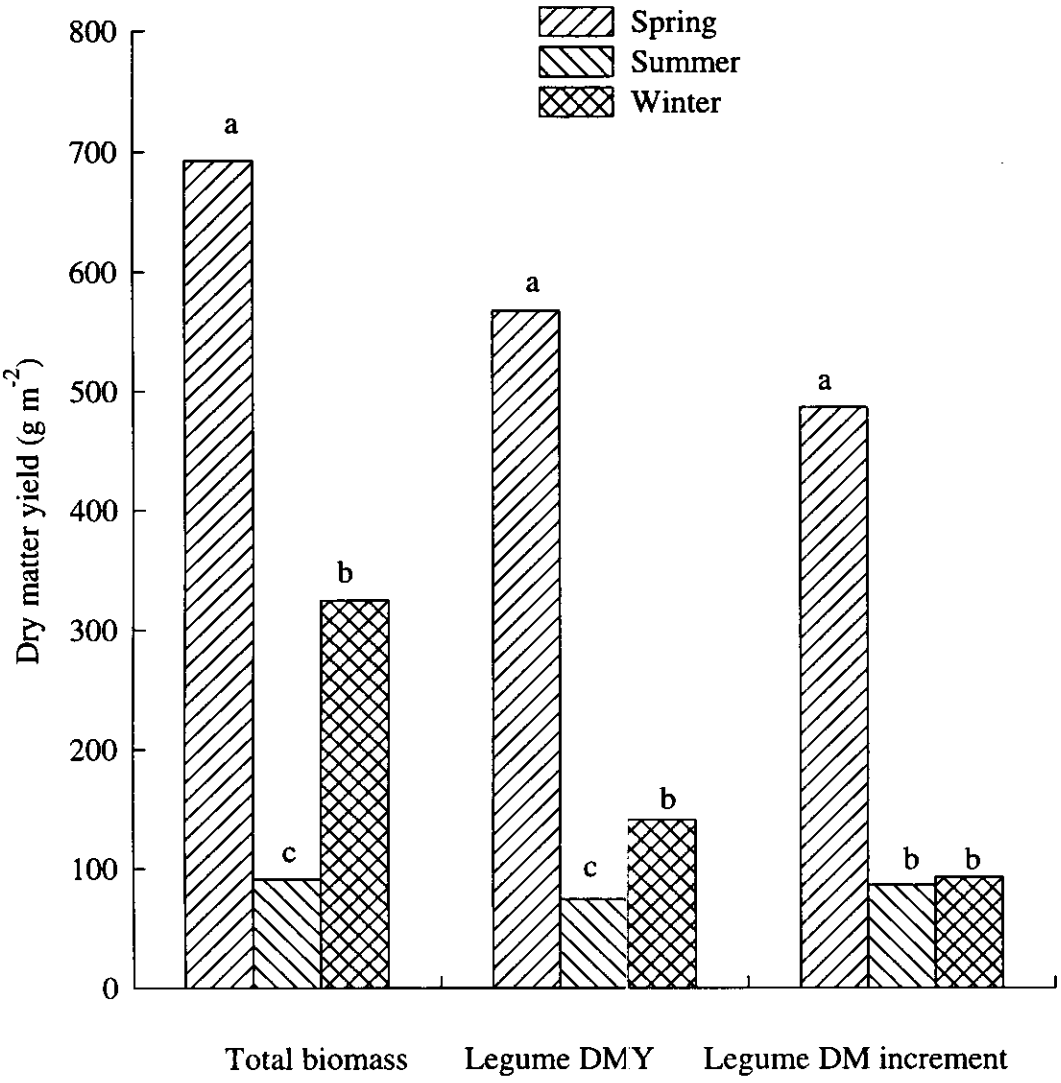

Figure 4.6. Seasonal total biomass, legume dry matter yield and legume DM increment in spring 1994, summer and winter 1995. Bars with the same letter within a paramenter are not significantly different at LSD ($P \leq 0.05$).

Table 4.15. Dry matter yield in above-ground live and dead components from legume and control plots in July 1995.

Component	DMY ($g\ m^{-2}$)	
	Legume plot	Control plot
Live dry matter	332.1aA	140.0bB
Dead dry matter	252.4aA	247.0aA
Total dry matter	584.5A	387.0B

Values followed by the same small letter in a column and capital letter in a row are not significantly different by linear contrast for differences between live and dead dry matter component, and differences between legume and control plot, respectively.

4.2.1.2.2. Root and ecosystem dry matter yield

Root DMY decreased with increase soil depth (Figure 4.7). About 87% of the root DMY in both the legume and control plot was found in the 0-10 cm depth. At each depth the RDMY did not differ significantly between the legume and the control plot (Table 4.16).

Both legume and control plot accumulated significantly more dry matter above ground (Table 4.17). The legume on the other hand, accumulated more dry matter above ground (77% of its total dry matter yield), compared to the control plot. However, there were no significant difference in the amount of root biomass and the total dry matter yield from the two plots.

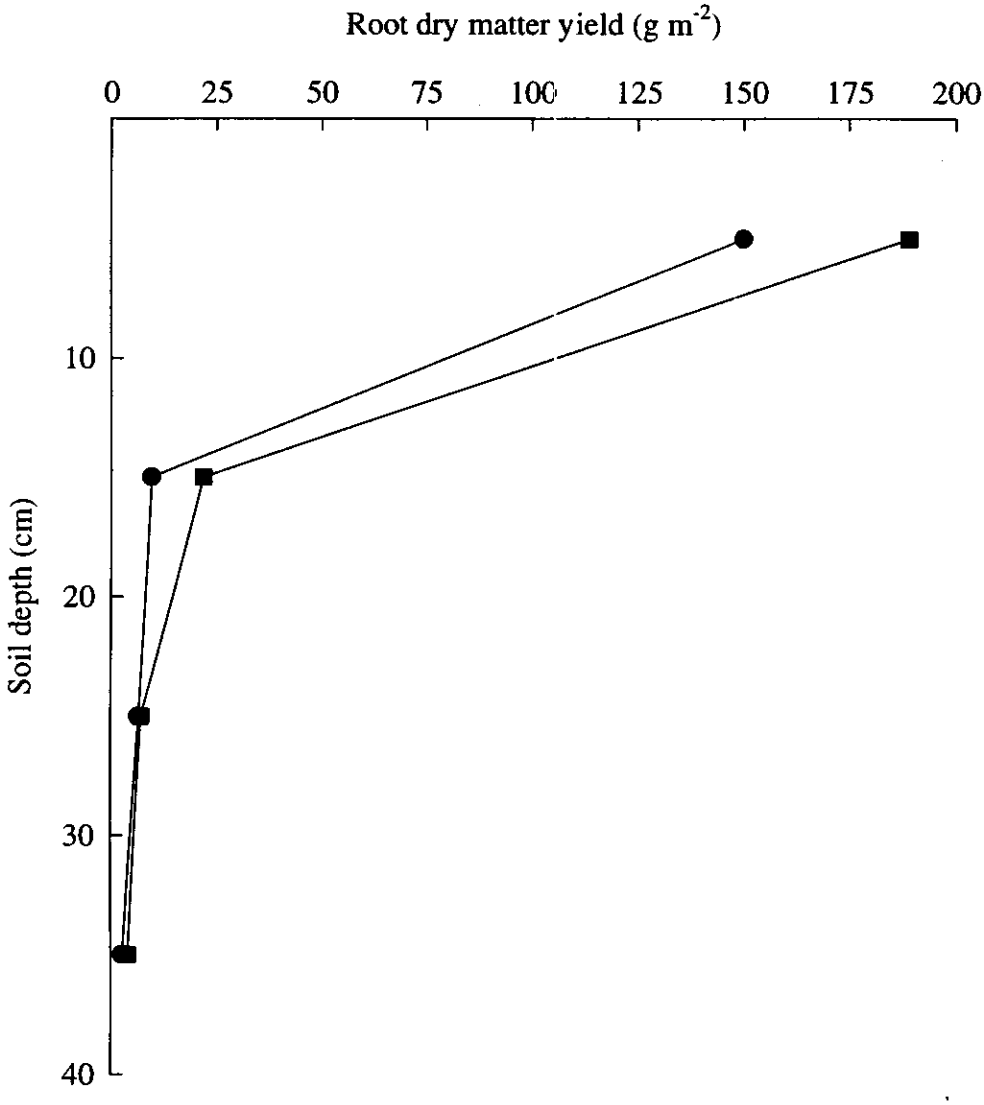

Figure 4.7. Root dry matter yield from legume (●) and control plots (■) in July 1995.

Table 4.16. Log-transformed values for root dry matter in legume and control plots in July 1995.

Soil depth (cm)	Log of RDMY				
	Legume plot	Control plot	CV (%)	Sig*.	SE
0-10	5.0	5.2	7.6	NS	0.39
10-20	2.3	3.1	25.0	NS	0.66
20-30	1.9	2.0	43.0	NS	0.83
30-40	1.0	1.4	112.20	NS	1.3

Sig.* = Significance
NS = Not significant at LSD (P≤0.05)

Table 4.17. Comparison of above- and below-ground plant dry matter from legume and control plots in July 1995.

Component	Dry matter ($g\ m^{-2}$)	
	Legume plot	Control plot
Above-ground	584.5aA	387.0aB
Below-ground	171.8bA	226.1bA
Total	756.3A	613.1A

Values followed by the same small letter in a column or capital letter in a row are not significantly different by linear contrast for differences between above- and below-ground components from each plot and differences between legume and control plot, respectively.

4.2.2. Nitrogen concentration and yield in dry matter

4.2.2.1. Seasonal nitrogen concentration and yield in legume above-ground biomass

Nitrogen concentration was significantly different at all sampling dates with the highest value in spring-summer (4.2%) and the lowest in winter-summer (2.2%) (Table 4.18).

The highest N yield was observed between winter and spring (July-November), constituting 63% of the annual N yield (Table 4.19). There were no significant seasonal differences in amounts of N yields in the period between spring and summer (November-February) and between summer and winter (February-July).

Table 4.18. Seasonal and annual mean nitrogen concentration in the above-ground legume biomass.

Season	Nitrogen concentration (%)
Spring	2.2c
Summer	4.2a
Winter	3.3b
Annual mean	3.2

Values followed by the same letter in a column are not significantly different at LSD ($P \leq 0.05$) for differences between seasons.

Table 4.19. Seasonal and annual N yield in above-ground legume biomass

Season	Nitrogen yield (g m^{-2})
Winter/Spring	11.4a
Spring/Summer	3.6b
Summer/Winter	3.0b
Annual N yield	18.0

Values followed by the same letter in a column are not significantly different at LSD ($P \leq 0.05$) for seasonal differences. Values are back transformed means.

4.2.2.2. Nitrogen accumulation in live and dead above-ground plant dry matter

Both legume and control plots accumulated significantly more N in the live biomass component constituting 66 and 58% of the total N in the dry matter from the respective plots (Table 4.20). In addition, the legume plot accumulated 7.6 g N m^{-2} more than the control.

Table 4.20. Nitrogen accumulation in the above-ground live and dead dry matter from legume and control plots in July 1995, after three years of legume growth.

Component	Nitrogen accumulated (g m^{-2})	
	Legume plot	Control plot
Live dry matter	7.8aA	2.5aB
Dead dry matter	4.1bA	1.8bB
Total dry matter	11.9A	4.3B

Values followed by the same small letter in a column or capital letter in a row are not significantly different by linear contrast for differences between live and dead component within each plot and differences between legume and control plot, respectively.

4.2.2.3. Root nitrogen yield

Root N yield decreased with increase in soil depth (Figure 4.8). About 97% of the N yield was found in the 0-10 cm depth. Nitrogen yield in the 10-20, 20-30 and 30-40 cm depths did not differ significantly between the legume and the control plot at each depth (Table 4.21).

Table 4.21. Log-transformed values for root N yield in legume and control plots in July 1995.

Soil depth (cm)	Log root N yield		CV (%)	Sig.*	SE
	Legume	Control			
0-10	0.9	0.9	40	NS	0.44
10-20	-2.8	-1.3	26	NS	0.65
20-30	-2.9	-2.6	26	NS	0.82
30-40	-4.3	-4.3	32	NS	1.37

Sig.* = Significance
NS = Not significant at LSD ($P \leq 0.05$)

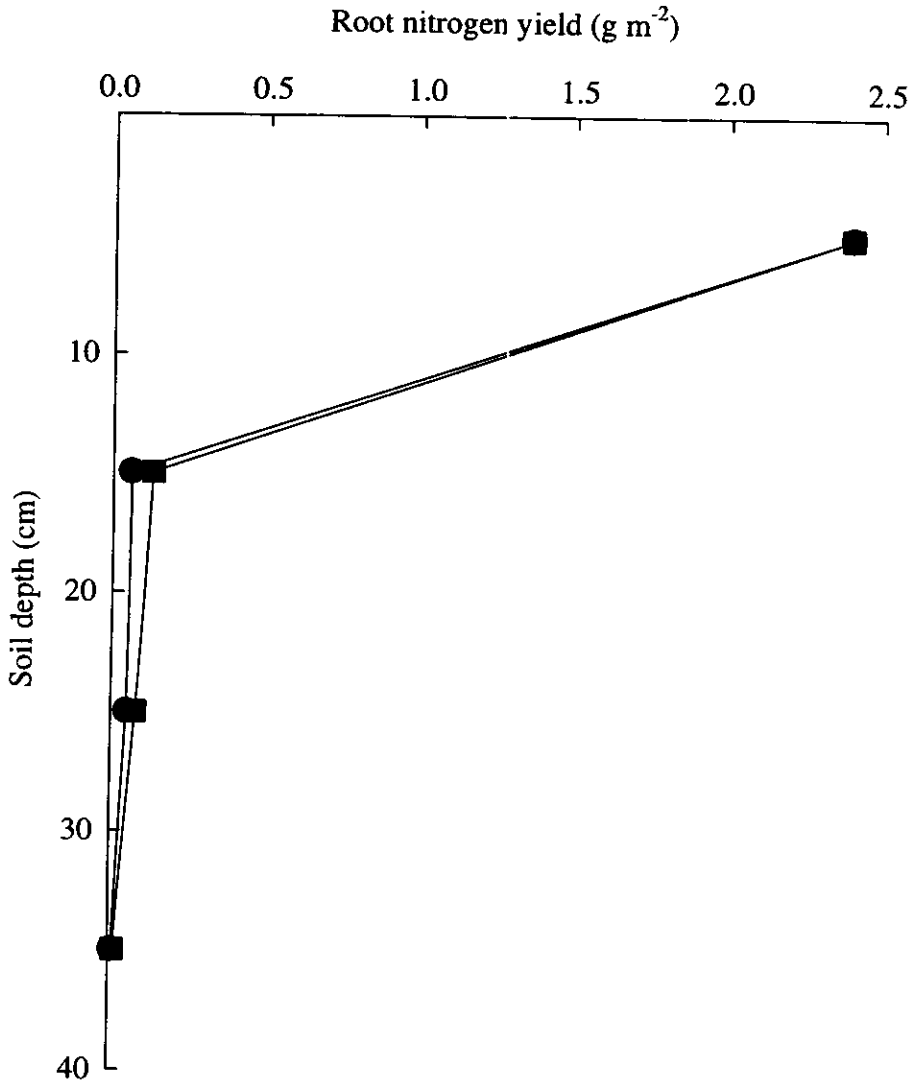

Figure 4.8. Root nitrogen yield from legume (●) and control (■) plots in July 1995.

4.2.3. Nitrogen distribution in soil and ecosystem

Total soil N decreased with increased soil depth in both the legume and control plots (Figure 4.9). No significant differences were observed in soil total N between the legume and control plots at each depth (Table 4.22).

Significantly more N, about 83 and 62% of the N in the plant dry matter was found in above-ground dry matter in the legume and control plot, respectively (Table 4.23). The legume plot accumulated significanlty more nitrogen in the total dry matter, (52%) than the control. However, the ecosystem total N did not differ significantly between the two treatments (Table 4.23).

Table 4.22. Log-transformed values for soil total N in legume and control plots in July 1995.

Soil depth (cm)	Log soil total N		CV (%)	Sig.*	SE
	Legume plot	Control plot			
0-10	4.7	5.0	10.5	NS	0.51
10-20	3.6	3.2	16.0	NS	0.55
20-30	2.5	3.1	34.7	NS	0.95
30-40	1.7	2.9	30.3	NS	0.67
40-50	0.7	1.8	68.6	NS	0.81

Sig.* = Significance
NS = Not significant at LSD ($P \leq 0.05$)
Soils were dried at 70°C

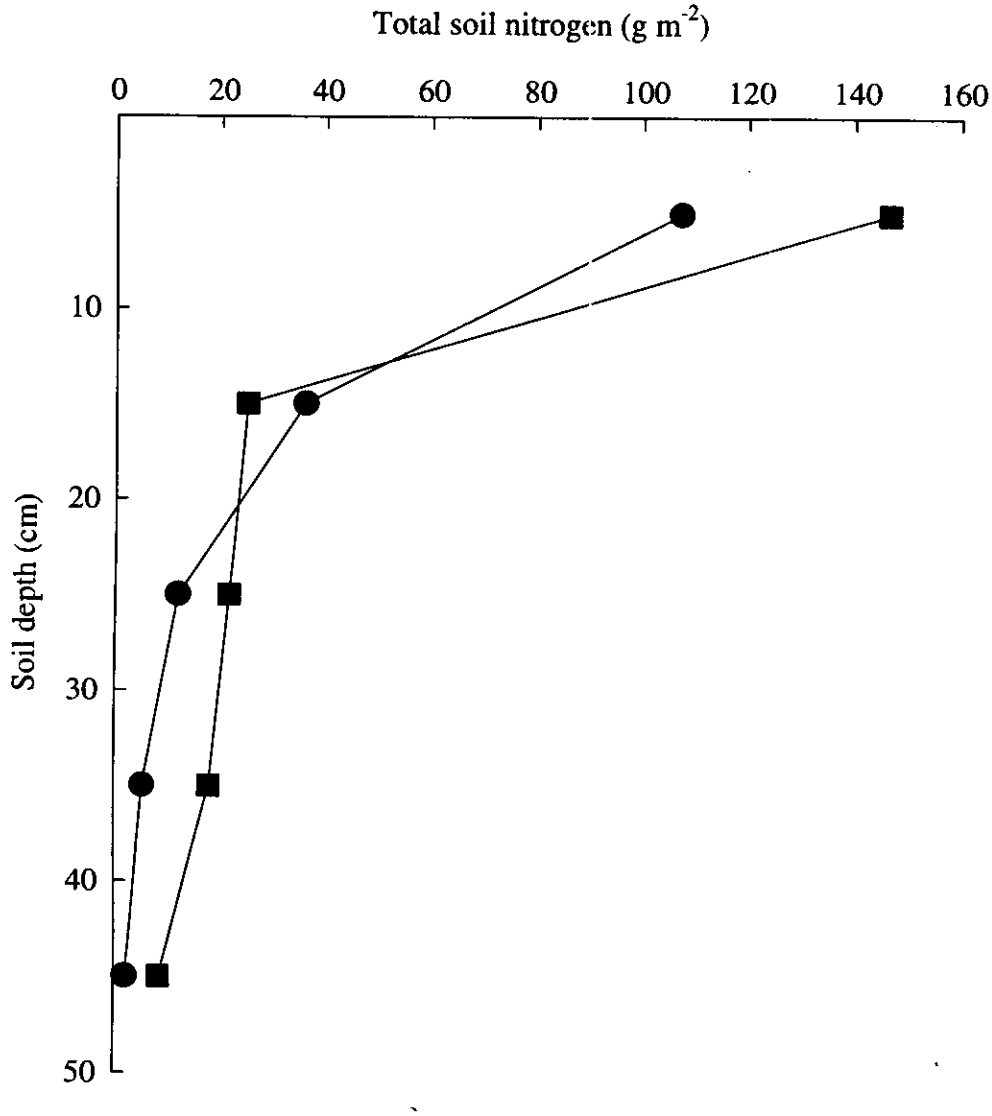

Figure 4.9. Total soil N at different soil depths in legume (●) and control (■) plots July 1995.

Table 4.23 Total N in above- and below-ground dry matter, and in the top 80 cm soil in July 1995.

Component	Nitrogen ($g\ m^{-2}$)	
	Legume plot	Control plot
Above-ground DM	11.9aA	4.3aB
Below-ground DM (0-40cm)	2.5bA	2.6bB
Total dry matter	14.4A	6.9B
Soil (0-80cm)*	169.7A	236.0A
Total ecosystem N	190.2A	242.8A

Values followed by the same small letter in a column or capital letter in a row are not significantly different by linear contrast for differences between above- and below-ground components from each plot, and differences between legume and control plot, respectively.

*Soils were dried at 70°C

4.2.4. Biological N_2 fixation in legume biomass

The proportion of N_2 derived from the atmosphere (%Ndfa) was highest in the period between winter and spring, and lowest between spring and summer (November-February) (Table 4.24).

The highest N_2 fixation was observed between winter and spring (July-November), constituting 73% of the annual N_2 fixation (Table 4.24).

Table 4.24. Proportion of N derived from the atmosphere (%Ndfa) and N_2 fixation in the legume above-ground biomass.

Parameters	Season			
	Winter-Spring	Spring-Summer	Summer-Winter	Annual mean
%Ndfa	87.2A	35.6C	75.8B	66.2
N fixed (g m^{-2})	9.7A	1.3C	2.3B	13.3

Values followed by the same letter in a row are not significantly different at LSD ($P \leq 0.05$) for seasonal differences. N-fixation values are back transformed means.

4.3. Prediction of nitrogen fixed by the legumes

In order to explore the relationships between the amount of N_2 fixed and the dry matter increment (DMI) and N concentration in the above-ground legume biomass, both non-stepwise and stepwise regressions were conducted utilising data from Experiment 1. Results from the non-stepwise regression showed that the r^2 value was 0.96 and was significant. Initial inspection of the data showed that logarithmically transformed data for N_2 fixed were more closely related to logarithmically transformed dry matter increment (DMI) than the non-transformed data.

The first variable entered for the stepwise regression was log DMI of legumes and the N concentration was entered at the second step. Both relationships were highly significant. The equations were:-

Step 1

Log N fixed = -4.0 + 1.11(Log DMI legume) $r^2 = 0.92$.....4.1

Step 2

Log N fixed = -4.97 + 1.1(Log DMI legume) + 0.29(%N legume).....4.2

$r^2 = 0.98$

where:-

r^2 = coefficient of determination

N fixed = amount of nitrogen fixed (g m^{-2})

DMI legume = legume dry matter increment

%N legume = N concentration in the above-ground biomass.

These close relationships suggest that it might be possible to estimate N_2 fixation from DMI and N concentration. Data from Experiment 2 was used as an independent test of equation 4.2. It was found that the equation predicted N_2 fixation well between winter and spring, and summer and winter periods when %Ndfa values were high, but not between spring and summer, when the %Ndfa was low (Table 4.25). The equation tended to overestimate N-fixation values.

Table 4.25. Comparison of N_2 fixation estimated by ^{15}N isotope dilution technique and predicted fixation from the regression equation, using data from Experiment 2.

Season	Predicted N fixed (g m^{-2})	Observed N fixed (g m^{-2})	% difference	% Ndfa
Winter/Spring	12.5	9.7	21.0	87
Spring/Summer	2.7	1.3	52.0	35
Summer/Winter	2.9	2.3	21.0	76

4.4. Estimation of N_2 fixation by Acetylene reduction assay (ARA)

4.4.1. Experiment 1. Nitrogen fixation by *Lotus*, *Dorycnium* and *Lathyrus* based on ARA method

In comparing the species, *Dorycnium* showed the highest N_2 fixation activity between spring and summer (February sampling) and the lowest between summer and winter (July sampling) (Table 4.26). Between winter and spring (November sampling), the species showed no significant differences in the amount of N_2 fixed. While *Lotus* and *Lathyrus* showed their lowest N_2 fixation between spring and summer (February sampling), *Dorycnium* showed no significant seasonal differences.

Table 4.26. Amount of N_2 fixed, estimated from acetylene reduction assay after 30 minutes root incubation with acetylene (Experiment 1).

Species	Nitrogen fixed (g m^{-2})			Annual
	Winter/Spring	Spring/Summer	Summer/Winter	N_2 fixation
Lotus	1.66aA	0.08bB	5.31aA	8.0a
Dorycnium	2.84aA	0.78aA	0.73bA	5.5a
Lathyrus	0.61aB	0.08bC	7.32aA	8.02a
CV (%)	65	54	70	41

Values followed by the same small letter in a column or capital letter in a row are not significantly different at LSD ($P \leq 0.05$) for differences between species and differences between seasons, respectively. Values are back transformed means.

4.4.2. Experiment 2: Estimation of N_2 fixation in the *Ornithopus, Lotus* and *Trifolium* sward based on ARA method

Significantly more N_2 was fixed between winter and spring (November sampling) (Table 4.27). This fixation comprised 56% of the total annual fixation. However, no significant differences were observed in the amount of N_2 fixed between spring and summer (February sampling) and summer and winter (July sampling) (Table 4.27).

Table 4.27. Amount of N_2 fixed, estimated from acetylene reduction assay after 30 minutes root incubation with acetylene (Experiment 2).

Season	Nitrogen fixed (g m^{-2})
Winter/Spring	5.7a
Spring/Summer	1.6b
Summer/Winter	2.8b
Annual mean N fixation	10.1

Values followed by the same small letter in a column are not significantly different at LSD ($P \leq 0.05$) for differences between seasons. Values are back transformed means.

CHAPTER 5

DISCUSSION

5.1. Above-ground plant dry matter dynamics

5.1.1. Above-ground biomass yield

Legume productivity and persistence varied with season. *Dorycnium* generally showed higher biomass than *Lotus* and *Lathyrus* at all sampling dates. Its seasonal above-ground biomass ranged between 420 g m $^{-2}$ in summer to about 680 g m^{-2} in spring (Table 4.3). *Dorycnium* is a woody perennial with a deep tap root (Sheppard and Douglas, 1986; Wills *et al.* 1989) which renders it moderately to highly tolerant to soil moisture deficit (Chapman *et al.* 1989; Woodman *et al.* 1992; Douglas and Foote, 1994). This tolerance and its woody nature probably led to its high biomass on this sandy site. The woody nature probably also contributed to its persistence even in the dry summer when the other species showed significantly low biomass (Table 4.3).

Lotus showed the lowest seasonal biomass (Figure 4.1). It had a total annual DMY increment of about 200 g m^{-2} (Figure 4.2). This amount was lower than that reported by West *et al.* (1988), of 300-500 g m^{-2} in a grazing trial, as understorey in 5-6 years radiata pine grown at 150-600 stems ha^{-1}. The higher yield in their study may be attributed to the application of superphosphate at the time of sowing since *Lotus* is P-responsive (West *et al.* 1988). On the other hand a very high productivity may compete with trees for nutrients and moisture. In forests where P is applied for tree growth, this should be able to benefit *Lotus*. Where P is not normally applied, there would be an additional cost of P topdressing to boost production. Schoeneberger *et al.* (1989) also recommended that where N is limiting, a starter N should be applied to enable legumes to establish until nodule formation and functioning commences.

Lathyrus in this study was observed to die back in summer and to begin new growth in spring. This may be the reason for the low and high biomass in summer and spring, respectively (Table 4.3). This pattern of growth has also been observed in *L. sylvestris* (Foster, 1990), a species closely related to *L. latifolius* in its growth pattern and rooting habit. *Lathyrus slyvestris* grows successfully in sand soils, tolerates salinity but not salt spray (Foster, 1990). Thus before *L. latifolius* can be introduced for wide spread planting at Woodhill it would be important to monitor its performance on sites that are prone to salt spray.

Annually the legumes in experiment 1 constituted between 60% and 87% of the total biomass. This may be an indication that up to about four years of growth, the legumes were capable of competing with other plant species in the ecosystem. In the past lupin succeeded on the sand dunes because it grew faster than developing weed population (Gadgil *et al.* 1986). Legumes could also reduce weed incidence. With herbaceous legumes this may in turn reduce the risk of ground fire as well as the cost of silvicultural operations.

Total biomass yield of *Ornithopus, Lotus and Trifolium* in experiment 2 was highest in spring (Figure 4.6). This maybe associated with the favourable weather during this period which may have stimulated legume growth. The plant die back over summer period may explain the low biomass during this period.

In winter, *Lotus* in experiment 2 yielded 51% more biomass than that in experiment 1. This may have been as a result of better soil nutrition arising from decomposing *Ornithopus* above-ground biomass which contained 13 g N m^{-2} in the standing biomass in spring compared to 4.1 g N m^{-2} from *Lotus* in experiment 1. Also P fertiliser applied during legume establishment in experiment 2 may be another factor that led to a higher biomass, since *Lotus* is P responsive.

Dorycnium vegetative growth has been reported to begin in early spring (Wills and Douglas, 1984) with branches dying back after pods have ripened in late spring (Sheppard and Douglas, 1986). This pattern was observed in the present study where the legume showed a high leaf biomass in spring, which decreased towards the summer period.

Sheath (1981) observed that *Lotus* has an extensive underground rhizome system which expands in autumn and fragments in winter. West *et al.* (1988) reported that many plants appear in spring from rhizome growth in the late summer and autumn. The rhizome fragmentation possibly gave rise to more points for the growth of new shoots, giving rise to more biomass production in spring. The favourable weather conditions in spring probably led to a rapid growth of the new shoots in spring with a consequent marked increase in biomass (Figure 4.1). *Lotus* also does not withstand drought on sandy soils (Clarkson *et al.* 1991), this may be the reason for its low biomass yield in summer during this study.

Lotus has been reported to be less winter hardy (Smethan, 1973) but this was not observed in the present study where the spring and winter biomass were not significantly different from each other. However, at the winter sampling in this study, the autumn growth was also included in the harvest.

Due to its hairy nature, *Dorycnium* has reduced palatability (Rhys *et al.* 1988) and only the young shoots are palatable to grazing stock, being useful for autumn and early spring forage (Sheppard and Douglas, 1986). Reduced palatability may be an advantage under extreme drought conditions since this will limit grazing. Though the species has been reported to be fire resistance (Wills and Douglas, 1984; Sheppard and Douglas, 1986), the slow decomposition of its woody biomass could increase the fire risks in the forest compared to a more herbaceous species.

Lotus is sensitive to grazing management. Due to its slow regrowth potential, it is vulnerable to competition from companion species if grazing is severe (Sheath and Hay, 1989). The slow recovery from defoliation results from the removal of actively growing shoots and from a delay in establishing the shoot population (Sheath, 1981). *Lotus* has a high concentration of tannins in its foliage (Clarkson *et al.* 1991), though this protects animals from bloat, excess may impair digestibility. Schiller and Ayres (1993), observed that *Lotus* has a low nutritive value in winter. This suggests that the species may not be a good all-season fodder. Sheath (1981) suggested that grazing should be limited during late summer and autumn to improve the rhizomes spread. This limited grazing period coupled with the low nutritive value in winter means that *Lotus* may only be a useful fodder in spring.

Pure stands of *Lotus* may not offer optimum diet and a mixed stand may be preferred. The choice of the legume to be grown in combination with *Lotus* should be selected with care. For instance, white clover would not be suitable since it has been demonstrated that the yield from such a mixture would be less than from *Lotus* alone (Lowther, 1976; Scott and Lowther, 1980). This decrease in DMY was associated with the clover taking up P which would otherwise be available for the more productive *Lotus*. In view of this, a companion legume should not compete with *Lotus* in P-uptake.

Most *Lathyrus* species have desirable forage characteristics, but utilisation of most species is restricted by the presence of a non-protein amino acid (Foster *et al.* 1989). Over indulgence in eating *Lathyrus* causes Lathyrism, an intoxication in animals. Toxic properties become evident at about the time of seed formation (Allen and Allen, 1981). Palatability appears to be highest in the vegetative and early bud stages decreasing as plant flower and produce seeds (Foster, 1990). Although *Lathyrus* has not been involved in poisoning animals in New Zealand (Lambrechtsen and Douglas, 1986),

it may be a useful fodder between autumn and winter as this would avoid grazing during the seed producing stage.

Grazing over spring could reduce the reseeding rates of *Ornithopus* since the plant has flowers at the branch tips. This legume may need to be resown every year (Lautour and Rumball, 1986), and this will involve an extra cost of re-establishment. This may be avoided by restricting grazing in spring. *Ornithopus* should be a useful forage over winter and early spring if replanting is to be avoided.

For a legume to be a successful understorey in a forest, it should be shade tolerant. *Lotus* has been reported to have good growth and persistence under pine (Gadgil *et al.* 1986; West *et al.* 1988; Fraser and Keogham, 1989). *Lathyrus* has also been reported to be shade tolerant (Foster, 1990). Good nodulation is dependent upon good rate of photosynthesis (Allison, 1973). Under shade conditions, a decline in host photosynthesis occurs (Gallon and Chaplin, 1987). This in turn limits the amount of photosynthate available for energy demanding N_2 fixation process. Exposure to shade has also been observed to significantly decrease root length and consequently nodule number and weight (Sprent, 1983). These factors lead to low rates of N_2 fixation.

Studies carried out on shade tolerance indicated that this is an important characteristic of the understorey in order to extend the period of N_2 fixation throughout canopy closure of the upper-storey (Turvey and Smethurst, 1983). There may be need to monitor changes in N_2 fixation potential of the legumes studied under shade since N inputs should continue even after crown closure. This will improve nutrition and maintain stand productivity (Turvey and Smethurst, 1983; Baker *et al.* 1988; Beets and Madgwick, 1988).

The ability of legumes to grow with other companion plants in the ecosystem is an important characteristic especially during the initial artificial succession stage. In the

past, marram grass grew as a companion crop and acted as a reserve for fixed N_2 when young pine plants were not able to utilise all the N (Gadgil, 1977; Mead and Gadgil, 1978; Gadgil, 1982). Small pine trees could not reach nor utilise all the N released by lupins, but marram grass with its underground network of roots and rhizomes was able to absorb N that otherwise may have been leached. In another study, Woods *et al.* (1992) reported that weeds increased ecosystem N-uptake by plant biomass and therefore improved N retention on site. It is hoped that the legumes being screened in this study will grow together with marram grass, which is the first crop to be introduced in the reclamation of uncolonised coastal sand in order to stabilise the mobile sand and turn the fragile ecosystem to a productive pine plantation.

Companion crops could also act as sink for soil mineral N which would prevent the depression in N_2 fixation that may result from a build up in soil inorganic N levels. It is also of great importance for the legumes to grow with grasses since the latter have more roots and fine rootlets that forage for P (Barea *et al.* 1989). This P could later be made available to the legumes and help in N_2 fixation which is P-dependent.

The main species growing in companion with the legumes studied included yorkshire fog (*Holcus lanatus* L.), sweet vernal grass (*Anthoxanthum odoratum* L.) and pampas grass (*Cortaderia selloana* L.). In some plots, the pampas grass occupied a large portion of the legume plots. This may have introduced variability among replicates due to competition for light, moisture and nutrients between legumes and grass.

Lathyrus slyvestris has a dense vegetative growth that enables it to effectively compete with grasses, for the same reason it may not be a suitable companion for slow growing trees (Foster, 1990). *Lathyrus latifolius* has a similar growth habit as *Lathyrus slyvestris*, which may enable this legume to suppress pampas grass; a weed that posses a lot of competition to the pine trees on this site. However if it is adapted as an

understorey crop there would be need to come up with a management protocol regarding how long the legume should be allowed to grow before pine tree establishment, the best method of suppressing the legume either by herbicide treatment, crushing or grazing, to ensure continuous growth of the legume crop as well as minimise competition with the young pine seedlings.

Lathyrus latifolius requires little management once established. Being a perennial with a large root system the species can survive for a long period of time regardless of seed removal. *Lathyrus slyvestris* has been reported to persist for over 50 years (Foster, 1990). *Lathyrus* species are usually rhizomous and in 4-5 years develop adequate root system that enable them to persist regardless of summer drought or soil fertility (Lambrechtsen and Douglas, 1986). If *Lathyrus* can thus survive over a period of pine tree rotation, this would save on the cost of re-establishment unlike an annual such as *Ornithopus* which may require replanting every year.

5.1.2. Above-ground dry matter accumulation

The high accumulation above-ground dead dry matter by *Dorycnium* was probably due to its woody nature whose relatively low N concentration (0.6%) compared with those of *Lotus* (1.3%) and *Lathyrus* (1.4%), probably led to slow decomposition rates. The build up of dead dry matter may increase fire risks, but may also conserve soil moisture by reducing soil moisture evaporation. Turvey and Smethurst, (1980) suggested that it may be advisable to have herbaceous legumes rather than woody ones as understorey to provide rapid litter decomposition. *Lathyrus* being a high productive non-woody legume with a high N concentration in its above-ground dry matter may have a rapid litter decomposition, preventing a build up of dead material on

the forest floor thus reducing fire risks. A higher C:N ratio in legumes promotes N-cycling, which may carry the risk N losses from the ecosystem (Steele and Vallis, 1988).

5.2. Nitrogen accumulation in plant dry matter and soil profile

At Woodhill, marram/lupin association accumulated about 440 kg ha^{-1} of N in biomass and litter over a period of 5 years, an amount equivalent to that required by the first crop of radiata pine (Gadgil, 1983). In the present study *Lotus, Dorycnium* and *Lathyrus* accumulated 64, 190 and 138 kg N ha^{-1}, respectively, while the mixed understorey of *Ornithopus, Lotus* and *Trifolium* (experiment 2) accumulated 119 kg N ha^{-1} in the above-ground biomass after three to four years of legume growth. The lower N accumulation values in the present study do not detract these legumes as possible replacement for the lupin, since the differences may have been caused by the varying establishment methods. In the marram/lupin association nitrogenous fertilisers added stimulated a vigorous growth of the marram grass, which with its fibrous root system was able to take up most of the N_2 fixed by the lupins and so preventing it against leaching losses. In the present study, fertiliser was not added during the legume establishment (Experiment 1). Also the main grasses during this study were pampas grass (*Cortaderia selloana* L), yorkshire fog (*Holcus lanatus*) and sweet vernal grass (*Anthoxanthum odoratum*), which may not be as efficient as marram grass in conserving N leaching losses. Pine trees planted one year after legume establishment, may have taken up and accumulated N which was not accounted for in this study. Madgwick (1985), estimated that pine trees on a fertile site could accumulate 6.9 kg N ha^{-1}, at a stocking of 2496 stems ha^{-1} and 131 kg N ha^{-1}, at a stocking of 2347 stems ha^{-1}, after two and four years, respectively. Pine trees at both experimental sites were 3 years old at

the time of sampling and may have accumulated quantities of N between these values in a site which was N deficient and trees were at a lower stocking, of 1000 stems ha^{-1}.

The absence of detectable N accumulation in the soil even after 3-4 years of legume growth, is in agreement with other observation in this area. Nitrogen was found to accumulate in the vegetation and litter layers rather than in the mineral soil (Gadgil, 1979; Gadgil *et al.* 1984; Dyck *et al.* 1988). This was attributed to the mineral soil having very low organic matter content. The nitrogenous products from the decomposition of the organic matter derived from the biomass, were not retained in the soil possibly due to leaching in free-draining sands. Soil N accumulation studies are not ideally suited in a clearfelled site where harvesting practices result in site disturbance and redistribution of organic matter causing large site variability. Another limitation stems from the very low and variable N levels especially at depths below 10 cm. Furthermore, small changes in total N levels are not detectable based on total N analysis without the use of a tracer (Goh, 1971).

Though the top 80 cm soil depth contained the largest N pool, averaging 71-90% of the ecosystem N (Table 4.12), it is probable that the legumes could play a very important role in N cycling by either storing N in the dead dry matter and roots or transferring it to a companion crop. This stored N could be released to the pine trees when they are able to utilise it, thus helping to reduce leaching losses. About half of the total N in the *Lotus* and *Lathyrus* accumulated in the root biomass (Table 4.12). The root could act as another storage for N, to be released when the roots die off due to senescence or defoliation from animal grazing.

5.3. Biological nitrogen fixation estimated by ^{15}N method

5.3.1 Proportion of N derived from the atmosphere (%Ndfa)

For *Dorycnium* and *Lathyrus*, the %Ndfa did not differ significantly across seasons. This is in agreement with the results of Barea *et al.* (1989) who working in a clover/rye grass mixture, found that the %Ndfa in clover shoots remained constant in all seasons. In contrast, Ledgard and Steel (1992) and Mansur (1994) working in a grazed pasture and an understorey, respectively, found significantly lower %Ndfa of the legume in a grass/legume mixture in summer and winter, respectively. This was attributed to the high and low temperatures, both of which reduce biological N_2 fixation (BNF).

Lotus in experiment 1 and all legumes in experiment 2 derived more N_2 from the atmosphere between winter and spring than between spring and summer (Tables 4.13 and 4.24). Neshein and Boller (1991) suggested that in a warm spring, legumes are stimulated to fix more atmospheric N_2 due to the competition for N from companion grasses. In the present study, the competition for soil N by other plants in the ecosystem and the favourable weather may have stimulated the legumes to depend on N_2 fixation in spring. In summer, the diversion of photosynthate to seed production may have hasten nodule senescence leading to the low fixation observed during this period.

In experiment 2, the %Ndfa was lowest between spring and summer (Table 4.24). This may have been caused by the presence of high levels of soil inorganic N possibly resulting from the decomposing biomass. Ledgard *et al.* (1987) and Simpson (1976) found high levels of soil inorganic N during summer. They concluded that this may have been as a result of root and above-ground biomass death in late spring, which might have been mineralised in summer when the temperatures were high and this possibly resulted in a release of inorganic N into the soil. Ball and Crush (1985) reported that N_2 fixation efficiency declines as N availability increases in the soil. Thus

increasing amount of soil inorganic N suppresses N_2 fixation. Mansur (1994) working in a grass/legume understorey suggested that during summer the N demand for grass was low leading to less competition for soil N between the grass and the legume. This makes the legume more dependent on soil N and in turn decreasing fixation.

Legumes derive part of their N from the atmosphere and a portion from the soil (LaRue and Patterson, 1981). For forest use it would be best to select legumes that derive much of their N from the atmosphere. This should maximise the benefits of N accretion to the site, while minimising the potential competition between the legume and the tree crop for soil N. In the present study, the legumes in experiment 1 derived between 81-98% of N from fixation, which suggests that they may be suitable understorey species as their demand for soil N is low.

5.3.2. Amount of biologically fixed nitrogen based on ^{15}N method

All legume species fixed more N between winter and spring in comparison to the period between spring and summer. Nitrogen fixation has been reported to be susceptible to environmental stress, especially low and high temperatures (Lie, 1981; Murphy and Ball, 1985) and moisture deficits (Sprent, 1973; Ball and Crush, 1985; Ledgard *et al.* 1988). Hansen *et al.* (1987) working with acacia species found increased N_2 fixation in the period between winter and spring. They associated this with the peak number of nodules found in the roots. The decrease in the summer N_2 fixation was associated with nodule senescence as a result of decreasing soil moisture. The low N_2 fixation observed between spring and summer in this study may be attributed to the high summer temperatures and the low rainfall conditions experienced during this period (Table 3.1). This moisture stress could have reduced nodule formation and subsequently N_2 fixation.

The significantly low amounts of N_2 fixed between summer and winter in *Lathyrus* compared to that fixed between winter and spring may have been caused by the low winter temperatures (Table 3.1). Low temperatures can disturb plant metabolism (Lie, 1981), nodule formation and rhizobia growth and infection, all of which reduce BNF. Local weather conditions can cause differences in N_2 fixation from year to year (Croychey, 1979; Wivstand *et al.* 1987). Croychey (1979), reported N_2 fixation of 90 and 160 kg N ha^{-1} yr^{-1} during consecutive years with dry and moist summer in a pasture under uniform management.

The amount of N_2 fixed has been reported to be closely related to DMY (Ledgard *et al.* 1987; Ledgard *et al.* 1988; Cadisch *et al.* 1989; Mansur, 1994; Goh *et al.* 1995). In the present study, these two parameters were highly correlated ($r^2 = 0.92$). It is probable that the high DMY increment of the legumes between winter and spring, was closely associated with the high amounts of N_2 fixed during this period. Although *Dorycnium* showed constantly low N concentrations in its above-ground biomass in comparison to *Lotus*, amounts of N_2 fixed by these two species did not differ significantly at any sampling date (Table 4.14), probably due to the compensatory effect of *Dorycnium* high biomass.

In experiment 2, the trend of N_2 fixation was similar to that of %Ndfa with the highest values between winter and spring and lowest between spring and summer. In spring, the legumes contained 13 g N m^{-2} in the standing biomass. By the following summer, all the legume biomass had died off. The decomposing biomass could thus have added a substantial amount of N in the soil, making the legumes more dependent on soil N in summer.

Jorgensen (1980), stipulated that legumes in forest management should provides at least 50-100 kg N ha^{-1} yr^{-1} for the first 3-5 years to provide an economic

return. In this study *Lotus, Dorycnium* and *Lathyrus* fixed 55, 71 and 214 kg N ha^{-1} yr^{-1}, respectively. *Lathyrus* fixed significantly more annual amounts of N which was probably the cause for its high mean N concentration and DMY annual increment. The legumes in experiment 2 fixed 133 kg N ha^{-1} yr^{-1}. Only *Lathyrus* fixed more N (214 kg N ha^{-1} yr^{-1}) than that fixed by lupin (160 kg N ha^{-1} yr^{-1}) in this ecosystem and probably will be capable of contributing N quantities required for the pine tree growth. Tree organs such as the stem could act as a store of N, reducing fixation by a feedback mechanism (Sprent, 1983). This is more likely to occur in *Dorycnium* since it has a woody, persistent stem.

The transfer of symbiotically derived N from the legumes to other plants in the ecosystem as well as fixation below-ground were not measured in this study, therefore rates measured could be considered as minimum.

5.4. Comparison of amounts of N_2 fixed estimated by ^{15}N and ARA methods

Estimation of N_2 fixation by ARA and ^{15}N methods generally showed low values between spring and summer than between winter and spring. This low N_2 fixation may be associated with high temperatures and low rainfall conditions experienced in summer both of which have a depressive effect on BNF. Environmental stress has been reported to cause sloughing of roots and nodules (Catchpoole and Blair, 1990; Danso *et al.* 1992), thus transferring N from the legumes to the soil pool and so leading to a reduction in N_2 fixation. High levels of soil N may also arise from the decomposing above-ground biomass that die off during summer.

Using ARA, the highest N_2 fixation for *Lathyrus* was observed in the period between summer and winter (Table 4.26). This contradicts the observation from the ^{15}N method where the N_2 fixation was significantly low in this period in comparison to the

period between winter and spring (Table 4.14). With the ARA, this species also showed lower N_2 fixation between winter and spring compared to that estimated between summer and winter. This observation is again in contrast to that from the ^{15}N method, where the species showed higher N_2 fixation between winter and spring. The higher and lower values from the ARA may be a reflection of a corresponding high and low nodule activity on the day of sampling as a result of weather condition at the time of the assay, other than a seasonal activity.

Though the absolute values estimated by ARA were variable, *Dorycnium* showed no significant seasonal differences in N_2 fixation unlike in the ^{15}N method, where the amount of N_2 fixed between winter and spring was significantly higher than that fixed between spring and summer. The ARA method also did not show any significant differences in the N_2 fixation among legume species between winter and spring period as well as in total annual N_2 fixation, unlike the ^{15}N dilution technique where *Lathyrus* showed significantly higher N_2 fixation.

In experiment 2, though the values reported using the ARA method were lower than those from the ^{15}N method, the same seasonal trends were observed using the two methods. Legumes showed a higher N_2 fixation between winter and spring possibly due to the warmer weather conditions experienced in spring. The low N_2 fixation between spring and summer, and summer and winter estimated by ARA may have been caused by a depressive effect of high and low temperatures in summer and winter, respectively. In both experiments, ARA generally showed lower values for N_2 compared to ^{15}N method. Various factors may be attributed to under-estimation of N_2 fixation by ARA.

For instance, the errors inherent in the ARA method which include inaccuracy due to extrapolation after a short period of incubation may have introduced a substantial amount of bias (Goh *et al.* 1978). In this study, a 30 minute incubation of root and soil

from a 0.00456m^{-2} area was extrapolated to fixation in a 1 m^{-2} area for 122 days. Attempts to avoid extrapolation error involved in the calculation of absolute amounts of N_2 fixation have been made by comparing acetylene reduction rates in relative terms at each sampling date. The acetylene reduction is then expressed as a % of the highest values recorded (Gadgil *et al.* 1986). This procedure was not followed in the present study since it would restrict comparison to a specific sampling date, making it impossible to carry out seasonal comparisons.

Use of the theoretical conversion ratio of 3:1 for C_2H_2/N_2 which was applied in the calculation of the N_2 fixed in this study may also have introduced errors in the estimation of N_2 fixation. Actual measurements of conversion ratio have ranged from 1.5 - 8.4:1 for pasture and food legumes and 1.6 - 4.8:1 for shrubs (Sprent and Silvester, 1973; Hardy *et al.* 1973; Hansen *et al.* 1987; Witty and Minchin, 1988). This ratio may vary due to soil N concentration and the climatic conditions. Witty and Minchin, (1988) advocated that if this conversion ratio is not determined in the field, ranking treatments on their ability to reduce C_2H_2 may be invalid. Treatments with highest C_2H_2 reduction activity may have a low electron allocation to N and consequently fix less N than others with a lower rate of C_2H_2 reduction. This was possibly another reason for the low ARA fixation values for *Lathyrus* between winter and spring and spring and summer.

Since excised nodules are less active than whole plants (Hardy *et al.* 1973; Smith and Hume, 1987) this may be another reason for the under-estimation of N_2 fixation using ARA in this study. When shoots are detached nitrogenase activity will eventually decline due to a decrease in photosynthate supply to the nodules necessary for sustained activity (Sinclair *et al.* 1976; Vessey, 1994). This may cause a decrease in electron flux through nitrogenase and hence lead to an under-estimation of N_2 fixation (Minchin *et al.* 1983). Insade (1991), found ARA could under-estimate N_2 fixation by

50%. The author suggested that ARA may, therefore, be only useful in measuring relative differences in N_2 fixation.

In this study, sampling was carried out throughout the day over a 3-4 day period at each sampling date. It was thus not possible to take into account diurnal variations which have great influence on nodule activity. Acetylene reduction accuracy has thus been restricted by the requirement for repeated assays to adjust for marked diurnal and seasonal variations. Diurnal variability may be overcome to some degree by sampling at the same time each day.

Lower acetylene activity have been reported for *Medicago truncatula* on the onset of flowering (Ruegg and Alston, 1978). This was associated with hormonal effects in the supply of assimilate to nodules. This may be another reason for the lower acetylene activity in spring for *Lotus* and *Lathyrus*, since the plants were flowering.

Bias may also be readily introduced if conditions of the days of acetylene reduction activity testing are not representative of the days of whole growth period. Martensson and Ljunggren (1984), suggested that ARA technique for estimating total N_2 fixation should be carried out on several occasions during plant development so as to obtain integrated values. Due to the destructive nature of this method it may not be practical to sample on seasonal basis, but to improve on the precision during sampling, smaller cores may be used (Sinclair *et al.* 1976). These will give a more representative sample, since more sampling per replicate can be done.

Major errors in the ARA are also likely to occur when the conditions of the assay are not carefully matched with the conditions under which N_2 fixation is taking place in the field. This may have occurred in the present study since the incubation jars were placed under a shade, which may not have reflected the soil temperatures at the time of sampling. Duplication of environmental conditions during incubation is difficult

under field conditions. This can be overcome by using *in situ* flow-through acetylene systems. Such systems have been used with some degree of success for the estimation of quantitative N_2 fixation (Denison *et al.* 1983; McNeil *et al.* 1989).

^{15}N dilution isotope studies though more expensive than the ARA gave more reliable estimates of N_2 fixation. The ^{15}N method integrates environmental and seasonal variations which was not possible with the ARA, which due to its destructive nature made it impossible to carry out repeated measurements during plant development. The assay as carried out in this study may not be used to estimate absolute values for N_2 fixation. It may also be incorrect to rank legumes on the basis of ARA values since the method is highly dependent on environmental conditions such as light, soil temperature and moisture at the time of sampling.

CHAPTER 6

CONCLUSION

Significant seasonal differences in biomass were observed for all legumes except *Dorycnium*. *Dorycnium* showed higher biomass than the other species studied at all sampling dates. This was associated with its woody nature which persisted in all the seasons. *Dorycnium* accumulated the highest quantity of dead plant dry matter making it unsuitable understorey legume as this could increase the fire risks in the forest.

Dorycnium and *Lathyrus* had an annual mean %Ndfa of 98 and 95%, respectively. Their %Ndfa values did not differ significantly with season. The legumes in experiment 2 showed the lowest %Ndfa between spring and summer. This also tended to be true for *Lotus* in experiment 1. This was associated the high temperatures and low rainfall conditions experiences in summer, both of which reduce BNF. The change in species dominance in experiment 2, may be another reason for the observed changes in %Ndfa between seasons.

The amount of N_2 fixed, estimated by the isotope ^{15}N dilution technique varied greatly with legume and season. *Lathyrus* fixed 214 kg N ha^{-1} yr^{-1}, an amount significantly higher than that fixed by *Lotus* (55 kg N ha^{-1} yr^{-1}) and *Dorycnium* (71 kg N ha^{-1} yr^{-1}). The higher N_2 fixation by *Lathyrus* was associated with its high DMY increments and N concentrations. The amount of N_2 fixed in each season depended on the persistence and the productivity of the legumes. All the legumes showed a higher biomass production between winter and spring. The high N_2 fixation observed between winter and spring maybe associated with the high biomass production in spring.

These results suggest that ranking above-ground legume productivity may be useful for selection between different herbaceous legumes. The seasonal growth pattern

and the competitive abilities of legumes would also be an important consideration for legume selection. However, if absolute N_2 fixation rates are required, then direct measurements should be carried out as this can vary with site, season and species.

There was a marked absence of N accumulation in the mineral soil even after 3-4 years of legume growth. Site variability and inherent difficulties in detecting total soil N, may have masked any differences in soil total N that may have been caused by the legumes.

The ARA in most cases under-estimated the N_2 fixation in comparison to ^{15}N isotope dilution technique. Results from ARA also suggested no clear pattern between legume fixation and season. These results were associated with the climatic conditions at the time of the assay. Satisfactory estimation of N_2 fixation by ARA may only be expected with *in situ* measurements as this could minimise errors inherent in ARA. The assay as carried out in this work may only be used as an indication of relative amounts of legume N_2 fixation, but is not suitable for providing absolute values for N_2 fixation. The ^{15}N isotope dilution technique is better placed than ARA as a method of estimating N_2 fixation in the field since it integrates environmental conditions.

From this study, *Lathyrus* appears to be the most suitable legume species for understorey growth in this ecosystem. Its high dry matter yield increments could support more livestock than the other species tested. Though *Lathyrus* is a rich fodder species, its use may be limited by the non-protein amino acids present, which could be toxic to animals if consumed in large quantities. Its high N_2 fixation in all seasons makes it less dependent on soil N. The high N-rich litter of *Lathyrus* would hasten decomposition of litter thus avoiding a build-up of dead material.

Future research

1. Further ^{15}N studies should be carried out to quantify N_2 fixation of the legumes studied under different climate conditions.
2. Legume persistence and N_2 fixation under shade condition should be investigated.
3. Research is also required to monitor competition between the legumes and young pine trees.
4. The nutritional quality of *Lathyrus* should be determined. Such studies should look at the non-protein amino acids levels, and their effects on animal performance.

Acknowledgments

I would like to thank my supervisors Dr Don Mead, Professor Kuan Goh, both of Lincoln University and Dr Ruth Gadgil of the New Zealand Forest Research Institute, Rotorua, for their great assistance in the production of this thesis. Special gratitude goes to Don Mead who offered me great support and advice throughout my two year study period in New Zealand.

I wish to register my appreciation to the New Zealand government for funding my study. Thanks also go to New Zealand Forest Research Institute and Carter Holt Harvey, Woodhill for their financial assistance which enabled me to carry out the field work.

Much gratitude also goes to Gaye Ridgen of Soil Science Department, Lincoln University who carried out all the soil and plant sample analysis. Sue Thomson, Eunice Anderson, Don Heffer and Dave Jack of Plant Science Department, Lincoln University are also acknowledged for the physical and moral support they offered me during my stay in New Zealand. I would also like to thank Alison, Doug, Helena and Ollie of New Zealand Forest Research Institute who assisted in the data collection and Acetylene Reduction Assay (ARA), both in the field and in the laboratory. I am also thankful to Harrison for his assistance in data analysis, valuable suggestions and the review of this thesis.

A very special thanks goes to my husband J.K. Ndambiri and our son Kariuki whose patience, understanding and support enabled me to complete my study.

REFERENCES

Allen, O.N. and Allen, E.K. (1981). *The leguminosae, a source book of characteristics, uses and nodulation,* University of Wisconsin Press, Madison, 812p.

Allison, F.E. (1973). *Soil organic matter and its role in crop production* Elsevier Scientific Publishing Company Amsterdam, 637p.

Baker, T.G., Oliver, G.R. and Hodgkiss, P.D. (1986). Distribution and cycling of nutrients in *Pinus radiata* as affected by past lupin growth and fertiliser. *Forest Ecology and Management* **17**: 169-187.

Ball, P.R. (1982). Nitrogen balances in intensively managed pasture systems In: *Nitrogen balances in New Zealand ecosystems* (P.W. Gandar ed.) DSIR, New Zealand, pp. 47-66.

Ball, P.R. and Crush, J.R. (1985). Prospects for increasing symbiotic nitrogen fixation in temperate grasslands *Proceedings of XV International Grassland Congress,* Tokyo, pp. 56-62.

Ballard, R. and Will, G.M. (1978). Past and projected use of fertiliser in New Zealand. *New Zealand Journal of Forestry Science* **8**: 15-26.

Barea, J.M., Azcon, R. and Azcon-Aguillar, C. (1989). Time-course of N_2 fixation (^{15}N) in the field by clover growing alone or in mixture with ryegrass to improve pasture productivity, and inoculated with vesicular-arbuscular mycorrhizal fungi. *The New Phytologist* **112**: 399-404.

Beets, P.N. and Madgwick, H.A.I. (1988). Above-ground dry matter and nutrient content of *Pinus radiata* as affected by lupin, fertiliser, thinning and stand age. *New Zealand Journal of Forestry Science* **18**: 43-64.

Berg, B., Muller, M.J. and Wessen, B. (1987). Decomposition of red clover *Trifolium pratense* roots. *Soil Biology and Biochemistry* **19**: 589-593.

Berg, P.J. and Smithies, C.W. (1973). Subsurface sowing of yellow lupin *Lupinus arboreus* for economical and rapid sand dune stabilisation at Woodhill forest. *New Zealand Journal of Forestry Science* **18**: 285-293.

Bergersen, F.J. (1970). The quantitative relationship between nitrogen fixation and acetylene reduction-assay. *Australian Journal of Biological Sciences* **23**: 1015-1025.

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

Catchpoole, D.W. and Blair, G.J. (1990). Forage tree legumes. II Investigation of nitrogen transfer to associated grass using split-root technique. *Australian Journal of Agricultural Research* **41**: 531-537.

Clarkson, N.M., Mears, P.T., Lowe, K.F. and Llord, D.L. (1991). Sustaining productive pastures in the tropics 8. Persistence and productivity of temperate legumes with tropical grasses. *Tropical Grasslands* **25**: 129-136.

Chalk, P.M. (1985). Estimation of N_2 fixation by isotope dilution:an appraisal of techniques involving ^{15}N enrichment and their application. *Soil Biology and Biochemistry* **17**: 389-410.

Chapman, H.P., Keogham, J.R., Cossen, C.G., Allan, B.E. and Littlejohn, R.P. (1989). Promising pasture species for drought prone slopes in South Island high country. *Proceedings of Agronomy Society of New Zealand* **19**: 77-84.

Cole, E.C. and Newton, M. (1986). Nutrients, moisture and light relations in five year old douglas fir plantation under variable competition. *Canadian Journal of Forest Research* **16**: 727-737.

Cookson, P., Goh, K.M., Swift, R.S. and Steele, K.W. (1990). Effects of frequency of application of ^{15}N tracer and rate of fertiliser nitrogen fixation by established mixed grass/clover pastures using ^{15}N enriched technique. *New Zealand Journal of Agricultural Research* **33**: 271-276.

Cox, J.E. (1977). Northland peninsula In: *Soil groups of New Zealand. Part 2. Yellow-brown sands* (V.E. Neall ed). New Zealand Society of Soil Science, Lower Hutt, pp. 18-47.

Crouchley, G. (1979). Nitrogen fixation in pasture VIII.Wairarapa plains dryland, Marterton. *New Zealand Journal of Experimental Agriculture* **7**: 31-33.

Danso, S.K.A. (1986). Review: Estimation of N_2-fixation by isotope dilution: An appraisal of techniques involving ^{15}N enrichment and their application-comments. *Soil Biology and Biochemistry* **18**: 243-244.

Danso, S.K.A. and Kumarasinghe, K.S. (1990). Assessment of potential sources of error in nitrogen fixation measurement by the nitrogen-15 isotope dilution technique. *Plant and Soil* **125**: 87-93.

Danso, S.K.A., Bowen, G.D. and Sanginga, N. (1992). Biological nitrogen fixation in trees in agroecosystems. *Plant and Soil* **141**: 177-196.

Dick, M.A. (1994). Blight of *Lupinus arboreus* in New Zealand. *New Zealand Journal of Forestry Science* **24**: 51-68.

Douglas, G.B. and Foote, A.G. (1994). Establishment of perennial species useful for soil conservation and as forages. *New Zealand Journal of Agricultural Research* **37**: 1-9.

Dyck, W.J., Beets, P.N., Will, G.M. and Messina, M.G. (1988). Nitrogen build up in sand-dune pine ecosystem In: *Forest site evaluation and long term productivity* (D.W. Cole and S.P. Gessel eds.) University of Washington Press, Seattle, pp. 107-112.

Edmeades, D.C. (1976). *The measurement of symbiotic nitrogen fixation in established pastures and some aspects of the nitrogen cycle in a sequence of pastures of increasing age*. A Thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy, Thesis, University of Canterbury, 257p.

Edmeades, D.C. and Goh, K.M. (1978). Symbiotic nitrogen fixation in a sequence of pastures of increasing age by ^{15}N dilution technique. *New Zealand Journal of Agricultural Research* **21**: 623-628.

Evans, P.S. (1973). The effect of repeated defoliation to three different levels on root growth of five pasture species. *New Zealand Journal of Agricultural Research* **16**: 31-34.

Foster, J.G., Cress Jr, W.D., Wright, S.F. and Hess, J.L. (1989). Intracellular localisation of neurotoxin 2,4-Diamino butyric acid in *Lathyrus slyvestris* L. leaf tissue. *Plant Physiology* **83**: 900-904.

Foster, J.G. (1990). Flatpea (*Lathyrus sylvestris* L.) a new forage species? A comprehensive review. *Advances in Agronomy* **43**: 241-313.

Fraser, W.J. and Keogham, J.M. (1989). The performance of several legume species on mine fallings in the Naseby forest. *Proceedings of Agronomy Society of New Zealand*. **19**: 125-129.

FRI (1977). Fertilisers or legumes in our forests? *What's new in forest research* No. 46 February 1977, Forest Research Institute, Rotorua, New Zealand, 4p.

FRI (1981). Legumes for infertile high-country soils. *What's new in forest research*. No. 93, Forest Research Institute, Rotorua, New Zealand, 4p.

Gadgil, R.L. (1971a). The nutritional role of *Lupinus arboreus* in a coastal sand dune forest. 2 The potential influence of damaged lupin plants on nitrogen uptake by *Pinus radiata*. *Plant and Soil* **34**: 575-593.

Gadgil, R.L. (1971b). The nutritional role of *Lupinus arboreus* in coastal sand dune forest. 111 Nitrogen distribution in the ecosystem before tree planting. *Plant and Soil* **35**: 113-126.

Gadgil, R.L. (1976). Nitrogen distribution in stands of *Pinus radiata* with and without lupin understorey. *New Zealand Journal of Forestry Science* **6**: 33-39.

Gadgil, R.L. (1977). Alternatives to the use of fertilisers In: *Use of fertilisers in New Zealand forestry* (R. Ballard. ed.), pp. 83-94.

Gadgil, R.L. (1979). The nutritional role of *Lupinus arboreus* in coastal sand dune forest. IV Nitrogen distribution in the ecosystem for the first 5 years after tree planting. *New Zealand Journal of Forestry Science* **9**: 324-336.

Gadgil, R.L. (1982). Biological nitrogen inputs to exotic forest In: *Nitrogen balances in New Zealand ecosystems* (P.W. Gandar and D.S. Bertaud eds.) Proceedings of a workshop on nitrogen balances in terrestrial ecosystem in New Zealand, Palmerston North, May 1980, pp. 123-129.

Gadgil, R.L. (1983). Biological nitrogen fixation in forests:Research and Practice in Australia and New Zealand In: *Biological nitrogen fixation in forest ecosystems: Foundations and applications* (G.J.C. Gordon and C.J. Wheeler eds.) Martinus Nijhoff/Dr. W. Junk, The Hague, pp. 317-332

Gadgil, R.L., Charlton, J.F.L., Sandberg, A.M. and Allen, P.J. (1988). Establishment of selected legumes in mid *Pinus radiata* plantations. *New Zealand Journal of Forestry Science* **18**: 210-220.

Gadgil, R.L., Sandberg, A.M. and Graham, J.D. (1984). *Lupinus arboreus* and inorganic fertiliser as nitrogen for *Pinus radiata* on a coastal sand. *New Zealand Journal of Forestry Science* **14:** 257-276.

Gadgil, R.L., Charlton, J.F.L., Sandberg, A.M. and Allen, P.J. (1986). Relative growth and persistence of planted legumes in mid-rotation radiata pine plantation. *Forest Ecology and Management* **14**: 113-124.

Gallon, J.R. and Chaplin, A.E. (1987). *An introduction to nitrogen fixation* Cassel Education Limited, Great Britain, 276p.

Giller, K.E. and Wilson, K.J. (1991). *Nitrogen fixation in tropical cropping system* CAB International Wallington, England, 244p.

Goh, K.M. (1971). Comparison and evaluation of methods for including nitrate in the total nitrogen determination of soils. *Journal of the Science of Food and Agriculture* **23**: 275-254.

Goh, K.M., Edmeades, D.C. and Robinson, B.W. (1978). Field measurement of symbiotic nitrogen fixation in an established pasture using acetylene reduction and ^{15}N method. *Soil Biology and Biochemistry* **10**: 13-20.

Goh, K.M., Ridgen, G.E. and Daly, M.L. (1995). Understorey biomass production and biological nitrogen fixation in an organic apple orchard in Canterbury, New Zealand. *Communications in Soil Science and Plant Analysis* **26**: 3261-3273.

Goodman, P.J. (1988). Nitrogen fixation, transfer and turnover in upland and lowland grass-clover swards, using ^{15}N isotope dilution. *Plant and Soil* **112**: 247-254.

Gordon, J.C. (1983). Silvicultural systems and nitrogen fixation In: *Biological nitrogen fixation in forest ecosystems:Foundations and Applications* (J.C. Gordon and C.T. Wheeler eds.) Martinus Nijhoff/Dr. W. Junk, The Hague, pp. 1-6.

Gordon, J.C. (1984). Biological dinitrogen fixation in temperate zone forestry:Current use and future potential In: *Advances in nitrogen fixation research Proceedings of the 5th symposium on nitrogen fixation*, Noordwijhout, The Netherlands August 28-September 13 1983 (C. Veegel and W.E. Newton eds.), pp. 15-22.

Haines, S.G., Haines, W. and Whire, G. (1978). Leguminous plants increase sycamore growth in Northern Albama. *Soil Science Society of American Journal* **42**: 130-132.

Hamilton, S.D., Chalk, P.M., Smith, C.J. and Hopman, P. (1991). Effect of nitrogen fertiliser rate on the estimation of N_2 fixation by isotope dilution. *Soil Biology and Biochemistry* **23**: 1105-1110.

Hamilton, S.D., Smith, C.J., Chalk, P.M. and Hopman, P. (1992). A model based on measurement of soil and plant ^{15}N enrichment to estimate N_2 fixation by soybean (*Glycine max* L. Merrill) grown in pots. *Soil Biology and Biochemistry* **24**: 71-78.

Hamilton, S.D., Hopmans, P.M., Chalk, P.M. and Smith, C.J. (1993). Field estimation of N_2 fixation by Acacia spp. using isotope dilution and labelling with ^{35}S. *Forest Ecology and Management* **56**: 297-313.

Hansen, A.P., Peoples, M.J., Gresshoff, P.M., Atkins, C.A., Pale, J.S. and Carrol, B.J. (1989). Symbiotic performance of super nodulating soybean (*Glycine max* (L) Merr.) mutant during development of different nitrogen regimes. *Journal of Experimental Botany* **40**: 000-010.

Hansen, A.P., Pate, J.S., Hansen, A. and Bell, B.T. (1987). Nitrogen economy of post-fire stands of shrub legumes in Tarah (*Eucalyptus marginata* forests of S. W. Australia). *Journal of Experimental Botany* **38**: 26-41.

Hardy, R.W.F., Burns, R.C. and Holsten, R.D. (1973). Application of acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry* **5**: 47-81.

Hauck, R.D. and Bremner, J.M. (1976). Use of tracers for soil and fertiliser nitrogen research. *Advances in Agronomy* **28**: 219-266.

Haynes, R.J. and Williams, P.H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in Agronomy* **49**: 119-199.

Haystead, A., Maljczuk, M. and Grove, T.S. (1988). Underground transfer of nitrogen between pasture plants infected with arbuscular mycorrhizal fungi. *The New Phytologist* **108**: 417-423.

Hendrickson, O.Q. and Burgess, D. (1989). Nitrogen fixing plants in a cut over lodgepole pine stand of southern British Columbia. *Canadian Journal of Forest Research* **19**: 936-939.

Herridge, D. and Peoples, M.D. (1989). Ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. *Plant Physiology* **93**: 495-503.

Herridge, D.F., Bergersen, F.J. and Peoples, M.B. (1990). Measurement of nitrogen fixation by soybean in the field using the ureide and natural ^{15}N abundance methods. *Plant Physiology* **93**: 708-716.

Insande, J. (1991). Regulation of nodule efficiency by the undisturbed soybean plant. *Journal of Experimental Botany* **42**: 687-691.

Jorgensen, J.R. (1980). Use of legumes in southeastern forest research In: *Southern silvicultural research conference proceedings*, Atlanta, Georgia U.S.A. November 6-7 1980 pp 205-211.

Kanehiro, Y., Walker, J.L. and Asghar, M. (1983). Special considerations for utilising tropical legumes in pastures: Edaphic factors In: *The role of Centrosema, Desmodium and Stylosanthes in improving tropical pastures* (R.J. Burt, J.L. Walker and M.W. Silvey eds.), Westview Press Inc., Boulder, pp. 197-219.

Lambrechtsen, N.C. and Douglas, G.D. (1986). Plant material for soil conservation Technical Note no.H8 Management and uses of *Lathyrus* spp In: *Plant material hand book for soil conservation Vol.2 Introduced plants* (C.W.S. van Kraayenoord and R.I. Hathaway eds.) Water and Soil Miscellaneous Publication, pp. 267-270.

LaRue, T.A. and Patterson, T.G. (1981). How much nitrogen do legumes fix? *Advances in Agronomy* **34**: 14-38.

Lautour, G. and Rumball, W. (1986). 'Grasslands koha' pink serradella (*Ornithopus sativus* Brot.). *New Zealand Journal of Experimental Agriculture* **14**: 465-467.

Ledgard, S.F. and Saunders, W.M.H. (1982). Effects of nitrogen fertiliser and urine on pasture performance and the influence of soil phosphorus and potassium status. *New Zealand Journal of Agricultural Research* **25**: 541-547.

Ledgard, S.F., Simpson, J.R., Frency, J.R. and Bergensen, F.J. (1985). Effect of reference plant on estimation of nitrogen fixation by subterranean clover using ^{15}N methods. *Australian Journal of Agricultural Research* **36**: 663-676.

Ledgard, S.F., Brier, G.J. and Littler, R.A. (1987). Legume production and nitrogen fixation in hill pasture communities. *New Zealand Journal of Agricultural Research* **30**: 413-421.

Ledgard, S.F., Brier, G.J. and Watson, R.N. (1988). New clover for Waikato dairy pasture: Establishment, production and nitrogen fixation during the first year. *Proceedings of New Zealand Grassland Association* **49**: 207-211.

Ledgard, S.F. and Steele, K.W. (1992). Biological nitrogen fixation in mixed legume/grass pasture. *Plant and Soil* **141**: 137-153.

Ledgard, S.F., Giller, K.E. and Bacon, P. E. (1995). Atmospheric N_2 fixation as an alternative N source In: *Nitrogen fertilisation in the environment* Marcel Dekker, Inc. New York pp 443-486.

Lie, T.A. (1974). Environmental effects on nodulation and symbiotic nitrogen fixation In: *The biology of nitrogen fixation* (A. Quispel ed.) North Holland Publishing Company, Amsterdam, pp. 553-582.

Lie, T.A. (1981). Environmental physiology of legume-rhizobium symbiosis In: *Nitrogen fixation* (W.J. Broughton ed.) Oxford University Press, New York, pp. 105-134.

Lowther, W.L. (1976). Comparison of Huia white clover and maku Lotus oversown separately or as a mixture. *Proceedings of New Zealand Grassland Association* **38**: 133-139.

Madgwick, H.A.I. (1985). Dry matter and nutrient relationships in stands of Pinus radiata. *New Zealand Journal of Forestry Science* **15**: 324-336.

Mansur, I. (1994). *Nitrogen uptake dynamics and biological nitrogen fixation in a silvopastoral system.* Master of Forestry Science, Thesis, Canterbury University, New Zealand 151p.

Marriott, C.A. (1988). Seasonal variation in white clover and nitrogen fixing (acetylene reducing) activity in a cut upland sward. *Grass and Forage Science* **43**: 253-262.

Martensson, A.M. and Ljunggren, H.D. (1984). A comparison between the acetylene reduction method, the isotope dilution method and the total nitrogen difference method for measuring nitrogen fixation in lucerne (*Medicago sativa* L.). *Plant and Soil* **81**: 177-184.

McColl, J.C. and Edmonds, R.C. (1986). Acetylene reduction by *Daviesea mimosoides* under eucalyptus. *Plant and Soil* **96**: 215-224.

McLaren, R.G. and Cameron, K.C. (1990). *Soil Science: An introduction to properties and management of New Zealand soils.* Oxford University Press, Auckland, 294p.

McNeil, A.M., Sheehy, J.E. and Drennan, D.S.H. (1989). The development and use of flow-through apparatus for measuring nitrogenase activity and photosynthesis in field crops. *Journal of Experimental Botany* **40**: 187-194.

Mead, D.J. and Gadgil, R.L. (1978). Fertiliser use in established radiata pine stands in New Zealand. *New Zealand Journal of Forestry Science* **8**: 105-134.

Mead, D.J. and Pritchet, W.L. (1975). Fertiliser movement in a slash pine ecosystem II N distribution after two growing seasons. *Plant and Soil* **43**: 467-478.

Minchin, F.R., Sheehy, J.E. and Muller, M. (1983). A major error in the acetylene reduction assay, Decrease in nodule nitrogenase activity under assay conditions. *Journal of Experimental Botany* **34**: 641-649.

Murphy, P.M. and Ball, P.R. (1985). Effect of early season nitrogen on nitrogen fixation in a ryegrass/white clover pasture *Proceedings of XV International Grassland Congress* Tokyo, pp. 460-461.

Nambiar, E.S.K. (1990). Interplay between nutrients, water, root growth and productivity in young plantations. *Forest Ecology and Management* **30**: 213-232.

Nambiar, E.S.K. and Nethercott, K.H. (1987). Nutrient and water availability to growth of young radiata pine plantations intercropped with lupins. *New Forestry* **1**: 117-134.

Neshein, L. and Boller, B.C. (1991). Nitrogen fixation by white clover when competing with grasses at moderately low temperatures. *Plant and Soil* **133**: 47-56.

O'Connell, A.M. and Grove, T.S. (1987). Seasonal variation in C_2H_2 reduction (N_2-fixation) in the litter layer of Eucalyptus forests of South-Western Australia. *Soil Biology and Biochemistry* **19**: 135-142.

Outcalt, K.W. and white, E.H. (1979). Understorey biomass and nutrients 2 years after timber harvest in northern Minnesota. Conference proceedings *Impact of Intensive harvesting on Forest Nutrient cycling* State University of New York, College of Environmental Science and Forestry, School of Forestry, Syracuse, New York.

Peoples, M.B., Faizah, A.W., Rerkasem, B. and Herridge, D.F. (1989a). Methods for evaluating nitrogen fixation by nodulated legumes in the field ACIAR Monograph Series No. 11, James Ferguson Pty Ltd. Hamilton, 76pp.

Peoples, M.B., Hebb, A.H. and Herridge, D.F. (1989b). Development of Xylem Ureide Assay for the measurement of nitrogen fixation by pigean pea (*Cajanus cajan* L.Millsp.). *Journal of Experimental Botany* **40**: 535-542.

Peoples, M.B. and Herridge, D.F. (1990). Nitrogen fixation in tropical and subtropical Agriculture. *Advances in Agronomy* **44**: 155-223.

Rennie, R.J. (1985) Nitrogen fixation in Agriculture in temperate regions In: *Nitrogen fixation research progress. Proceedings of 6th international symposium on nitrogen fixation* August 4-10 1985 (H.J. Evans, J.P. Bottomley and E.W. Newton eds.) Martinus Nijhoff Publishers, Dordrecht, pp. 659-665.

Restall, A.A. (1964). Sand dune reclamation on Woodhill forest. *New Zealand Journal of Forestry* **9**: 154-161.

Rhys, G.J., Smith, N. and Slay, M.W. (1988). Alternative forage species for Hawks Bay. *Proceedings of Agronomy Society of New Zealand* **18**: 75-80.

Rohini-Kumarasighe, M.K. and Nutman, P.S. (1979). The influence of temperature on root hair infection of *Trifolium parviflora* and *T. glomeratum* by root nodule bacteria 1. The effects of constant root temperature infection and related aspects of plant development. *Journal of Experimental Botany* **30**: 503-515.

Ruegg, J.J. and Alson, A.M. (1978). Seasonal and diurnal variation of nitrogen activity (acetylene reduction) in barrel medic (*Medicago truncatula* Gaertn.) *Australian Journal of Agricultural Research* **29**: 951-961.

Sarathchandra, S.U. and Upsell, M.P. (1981). Nitrogen mineralisation and the activity and population of microflora in a high producing brown loam under pasture. *New Zealand Journal of Agricultural Research* **24**: 171-176.

SAS Institute inc. (1990). SAS/STAT guide for personal computer, Version 6, SAS Institute Inc. Carry, NC.

Schiller, K.N. and Ayres, J.F. (1993). The effect of winter conditions on the nutritive value of *Lotus pedunculatus* cv. Grasslands Maku and *Trifolium repens* cv. Haifa. *Tropical Grasslands* **27**: 43-47.

Schoeneberger, M.M., Volk, R.J. and Davey, C.B. (1989). Factors influencing early performance of leguminous plants in forest soils. *Soil Science Society of American Journal* **53**: 1429-1434.

Scott, R.S. and Lowther, W.L. (1980). Competition between white clover 'Grasslands Huia" and *Lotus pedunculatus* 'Grasslands maku'. *New Zealand Journal of Agricultural Research* **23**: 501-507.

Sheath, G.W. (1980). Production and regrowth characteristics of *Lotus pedunculatus* cav. "Grasslands maku". *New Zealand Journal of Agricultural Research* **23**: 201-210.

Sheath, G.W. (1981). *Lotus pedunculatus:* An agricultural plant? *Proceedings of New Zealand Grassland Association* **42**: 160-170.

Sheath, G.W. and Hay, R.J.M. (1989). Overview of legume persistence in New Zealand In: *Persistence of forage legume Proceedings of a trilateral workshop held in Honolulu, Hawaii 8-22 July 1988* (G.C. Maten, A.G. Matches, R.F. Barnes, R.W. Brougham, R.J. Clements. and G.W.Sheath eds.) Madison, American Society of Agronomy, pp. 23-36.

Sheppard, J.S. and Douglas, G.D. (1986). Plant material for soil conservation, Technical Note No.H6. Management and uses of *Dorycnium* spp. In: *Plant materials Handbook for soil conservation Vol 2, Introduced plants* (C.W.S. van Kraayenoord and Hathaway eds.) Water and Soil Miscellaneous Publication No. 94, Wellington pp. 260-262.

Simpson, J.R. (1976). Transfer of nitrogen from the pasture legumes under periodic defoliation in a field environment. *Australian Journal of Experimental Agriculture and Animal Husbandry* **16**: 863-870.

Sinclair, A.G., Hannagan, R.B. and Risk, W.H. (1976). Evaluation of acetylene-reduction assay of nitrogen fixation in pastures using small soil core samples. *New Zealand Journal of Agricultural Research* **19**: 451-458.

Smethan, M.L. (1973). Pasture legumes and strains In: *Pasture and pasture plants* (R.H.M. Langer ed.) A.H. and A.W. Reed, Wellington, pp. 85-128.

Smith, D.L. and Hume, D.J. (1987). Comparison of assay methods for N_2 fixation utilising white bean and soybean. *Canadian Journal of Plant Science* **67**: 11-19.

Sprent, J.I. (1973). Growth and nitrogen fixation in *Lupinus arboreus* as affected by shading and water supply. *The New Phytologist*. **72**: 1005-1022.

Sprent, J.I. and Silvester, W.B. (1973). Nitrogen fixation by *Lupinus arboreus* grown in the open and under different aged stands of *Pinus radiata*. *The New Phytologist* **72**: 991-1003.

Sprent, J.I. (1979). *The biology of nitrogen fixing organisms* McGraw-Hill Book Company (UK) Limited, Maidhead 196p.

Sprent, J.I. (1983). Agricultural and horticultural systems:Implications for forestry In: *Biological Nitrogen fixation in forest ecosystems:Foundations and applications* (J.C. Gordon and C.T. Wheeler eds.) Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, pp. 213-232.

Sprent, J.I., Becana, M. and Sutherland, J.M. (1988). Optimising nitrogen fixation in legumes crops and trees In: *Nitrogen fixation: A hundred years after* (H. Bother, F.J. de Bruijin and W.E. Newton eds.) Gustav Fischer, Stuttgart, pp. 725-733.

Steele, K.W. and Vallis, I. (1988). The nitrogen cycle in pastures In: *Advances in nitrogen cycling in agricultural ecosystems* (Wilson, J.R. ed.) CAB International, Wallingford, pp 274-291.

Sutton, W.R.J. (1976). Comparison of alternative silvicultural regimes for radiata pine. *New Zealand Journal of Forestry Science* **6**: 350-356.

Ta, J.C. and Faris, M.A. (1987). Species variation in the fixation and transfer of nitrogen from legumes to associated grasses. *Plant and Soil* **98**: 265-274.

Thomas, R.C. and Mead, D.J. (1992a). Uptake of nitrogen by *Pinus radiata* and the retention within the soil after applying ^{15}N-labelled urea at different frequencies. 1. Growth response and nitrogen budgets. *Forest Ecology and Management* **53**: 131-151.

Thomas, R.C. and Mead, D.J. (1992b). Uptake of nitrogen by *Pinus radiata* and retention within soil after applying ^{15}N-labelled urea at different frequencies 2. Seasonal trend and processes. *Forest Ecology and Management* **53**: 153-174.

Turner, G.L. and Gibson, A.H. (1980). Measurement of nitrogen fixation by indirect means In: *Methods for evaluating biological nitrogen fixation* (F.J. Bergersen ed.) John Wiley and Sons Ltd, Chichester, pp. 111-138.

Turvey, N.D. and Smethurst, P.J. (1980). Biological and economic criteria for establishing understorey in pine plantation In: *Managing Nitrogen Economies of Natural and Man Made Forest Ecosystems* (R.A. Rummery and F.J. Hingston eds.) Perth CSIRO, Division of Land Resource Management, pp. 124-145.

Turvey, N.D. and Smethurst, P.J. (1983). Nitrogen fixing plants in forest plantation management In: *Biological nitrogen fixation in forest ecosystems* (J.C. Gordon and C.T. Wheeler eds.) Martin Nijhoff/Dr. W. Junk Publishers, The Hague, pp. 233-259.

van Kraayenoord, C.W.S. (1986). Plant material for sand dune stabilisation In: *Plant material hand book for soil conservation, vol. 1, Principles and practices. Water and Soil Miscellaneous Publication* No.93 (C.W.S. van Kraayenoord and R.L. Hathaway eds.) National Water and Soil Conservation Authority, Wellington, pp. 69-77.

Vessey, J.K. (1994). Measurement of nitrogenase activity in legume root nodules: In defence of acetylene reduction assay. *Plant and Soil* **158**: 151-162.

Viera-Vargas, M.S., Souto, C.M., Urquiaga, S. and Boddey, R.M. (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.

Vincent, J.M. (1974). Root nodule symbiosis with rhizobia In: *The biology of nitrogen fixation* (A. Quispel ed.) North Holland Publishing Company, Amsterdam, pp. 265-341.

Weldelken, W.J. (1974). New Zealand experience in stabilisation and afforestation of coastal sands. *International Journal of Biometeorology* **18**: 145-158.

West, G.G., Percival, N.S. and Dean, M.G. (1988). Oversowing legumes and grasses for forest grazing: Interim research results In: *Proceedings of the Agroforestry Symposium,* Rotorua, 24-27 November 1986 (P. Maclaren ed.), FRI Bulletin No. 139, pp. 203-220.

Whitehead, D.C., Buchan, H. and Hartley, R.D. (1979). Composition and decomposition of roots of ryegrass and red clover. *Soil Biology and Biochemistry* **11**: 619-628.

Will, G.M. (1978). Nutrient deficiency in *Pinus radiata* in New Zealand. *New Zealand Journal of Forestry Science*. **8**: 4-14.

Will, G.M. (1981). Use of fertilisers in New Zealand forestry operations in 1980. *New Zealand Journal of Forestry Science* **11**: 191-198.

Will, G.M., Dyck, W.J., Gadgil, R.L., Hunter, I.R. and Madgwick, H.A.I. (1980). Nitrogen in radiata pine forests in New Zealand In: *Managing Nitrogen Economies of Natural and Man Made Forest Ecosystems* (R.A. Rummery and F.J. Hingston eds.) CSIRO Division of Land Resource Management, pp. 146-152.

Will, G.M. and Hodgkiss, P.D. (1977). Influence of nitrogen and phosphorus stress on the growth and form of radiata pine stems and crowns. *New Zealand Journal of Forestry Science* **7**: 307-320.

Williams, M. (1993). Forest pathology in New Zealand: Lupin blight No.22 (P.D. Gadgil ed.) New Zealand Forest Research Institute, Rotorua, 3p.

Wills, B.J. (1986). Soil conservation plants for the semi-arid high country and rangelands of New Zealand In: *Rangelands: A resource under siege Proceedings of the 2nd International Rangeland Congress*, Aldelaide, Australia, 13-18 May 1984, pp. 309-310.

Wills, B.J. and Douglas, G. (1984). Canary clovers, Streamland No.32, National Water and Soil Conservation Authority Publication, Wellington 4p.

Wills, B.J., Begg, J.S.C. and Foote, A.G. (1989). *Dorycnium* species - two new legumes with potential for dry land pasture rejuvenation and resource conservation in New Zealand. *Proceedings of New Zealand Grassland Association* **50**: 169-174.

Witty, J.F. (1983). Estimating N_2 fixation in the field using labelled fertiliser. Some problems and solutions. *Soil Biology and Biochemistry* **15**: 631-639.

Witty, J.F. and Minchin, F.R. (1988). Measurement of nitrogen fixation by acetylene reduction assay. Myths and mysteries In: *Nitrogen fixation in the mediterranean agriculture* (D.P. Bech and L.A. Marteron eds.) Martinus Nijhoff, Dordrecht, pp. 331-334.

Witty, J.F., Rennie, R.J. and Atkins, C.A. (1988). ^{15}N addition methods for assessing N_2 fixation under field conditions In: *World crop: Cool season food legumes* (R.J. Summerfield ed.) Kluwer Academic Publishers, Dordrecht, pp. 716-739.

Wivstand, M., Martensson, A.M. and Ljunggren, H.D. (1987). Field measurement of symbiotic nitrogen fixation in an established lucerne ley using ^{15}N and an acetylene reduction. *Plant and Soil* **97**: 93-104.

Woodman, R.F., Keoghan, J.M. and Allan, B.E. (1992). Pasture species for drought-prone lower slopes in the South Island high country. *Proceedings of New Zealand Grassland Association* **54**: 115-120.

Woods, P.V., Nambiar, E.K.S. and Smethurst, P.J. (1992). Effects of annual weed on water and nitrogen availability to *Pinus radiata* trees in a young plantation. *Forest Ecology and Management* **48**: 145-163.

Zeihm, R.W., Pearson, H.A., Thurow, T.L. and Baldwin, V.C. (1992). Pine growth response to management of the subterranean clover understorey. *Agroforestry Systems* **20**: 267-274.

APPENDICES

Appendix 1. Full names of the legumes established in Experiment 1, show in Figure 3.1.

Ast cic	*Astragalus cicer*
Cor var	*Coronilla varia*
Dor hir	*Dorycnium hirsutum*
Lat lat	*Lathyrus latifolius*
Lot cor	*Lotus corniculatus*
Lot ped	*Lotus pedunculatus*
Lot ten	*Lotus tenuis*
Lup pol	*Lupinus ployphyrus*
Mel alb	*Melilotus alba*
Mel ind	*Melilotus indica*
Tri amb	*Trifolium ambiguum*
Tri dub	*Trifolium dubium*
Tri sub	*Trifolium subterranean*
Vic vil	*Vicia villosa*

Appendix 2. Calculation of the required labelled ammonium sulphate $\{(NH_4)_2SO_4\}$ fertiliser applied to ^{15}N treatment plots (TP).

Preparing a 30 atom $\%^{15}N$ enriched $(NH_4)_2SO_4$ from a 60.4% enriched $(NH_4)_2SO_4$ source,

Want to add 5.475 kg N ha^{-1} $year^{-1}$. This amount was split over three applications:-

5.475/3 = 1.825 kg N ha^{-1} $application^{-1}$, which is an amount equivalent to 182.5 mg N m^{-2}. For every $1m^2$ 10 ml aliquot is required

Each 10 ml aliquot of stock solution should contain 182.5 mg N. To fertiliser 13 plots 130 ml of stock solution is required.

10 ml aliquot has 182.5 mg N

So 130 ml will require 130/10 x 182.5 = 2.3725g N.

1 mole of N = 14.0067g

? mole of N = 2.3725g N.

2.3725g N x 1/14.0067 = 0.1693832 moles

A_1 and A_2 are the atom $\%^{15}N$ excess required in the experiment and that of the enrichment source respectively, while E and (1-E) are the proportion of the amount of the $(NH_4)_2SO_4$ from enriched and diluting source respectively.

$E = A_1/A_2$,

A_1 = (30 - 0.366) = 29.634

A_2 = (60.4 -0.366) = 60.034

E = 29.634/60.034 = 0.4936202

1-E = 1- 0.4936202 = 0.5063798

Moles needed from diluting source = 0.1693832 mole x 0.5063798 = 0.0857722

Moles needed from enriched source = 0.1693832 moles x 0.4936202 = 0.0836109

Mean atomic weight (μm) from dilution source

0.01(0.366 x 15 + 99.634 x 14.003) = 14.006649 g/mole

Mean atomic weight (μm)from enriched source

0.01(60.4 x 15 + 39.6 x 14.003) = 14.605188 g/mole

Moles from diluting source = 0.0857722 moles x 14.006649 = 1.201381 moles.

Moles from enriched source 0.0836109 moles x 14.605188 = 1.2211529 moles

Thus amount of N from dilution source = 2 x 14.006649/132.14 = 0.211997 gN/g $(NH_4)_2SO_4$

and the amount of N from enriched source = 2 x 14.605188/132.14 =0.2210562 gN/g$(NH_4)_2SO_4$

Amount to add from diluting source 1.201381/0.211997 = 5.66696g

Amount from enriched source 1.2211529/0.2210562 = 5.524173

Appendix 3. Calculation of the unlabelled $(NH_4)_2SO_4$ required for the plot surround (PS).

Amount of N to be added = 5.475 kg N /ha/ year

This is equivalent to 182.5 mg N m^{-2}

The mean weight of N = 14.0067g

The N content in every g $(NH_4)_2SO_4$ = 0.2119971,

Size of the treatment plots surround = 8 m^2

Amount of N needed for every PS = 182.5 mg N x 8 = 1460 mg

1460/0.2119971 = 6886.8866 mg

Every 10 ml aliquot solution contains 6886.8866 mg N

Therefore 130 ml should contain 130 x 6886.8866 mg/10 = 89.5295 g of $(NH_4)_2SO_4$

The amount of was dissolved in 260 ml of distilled water and so for every PS 20 ml of the aliquot was required

Appendix 4. Comparison of ^{15}N levels, %Ndfa and estimated amounts of N fixed using *Holcus lanatus* and *Anthoxanthum odoratum* as reference plants.

Plot	Holcus	Anthoxanthum	CV (%)
		^{15}N	
Lotus	0.6738A	0.8965A	17.8
Dorycnium	0.7143A	0.8612A	27.7
Lathyrus	0.6449A	0.8977A	19.9
Mixed legume sward	0.5788	0.6762A	16.7
		%Ndfa	
Lotus	96.8A	98.1A	1.1
Dorycnium	98.4A	99.1A	1.8
Lathyrus	97.3A	99.1A	1.8
Mixed legume sward	87.2A	91.6A	7.0
		Amount of N fixed (g m^{-2})	
Lotus	3.9A	4.0A	55.6
Dorycnium	3.3A	3.3A	55.8
Lathyrus	13.8A	14.1A	22.8
Mixed legume sward	9.44A	9.93A	16.7

Values followed by the same letter in a row are not significantly different for differences between ^{15}N, %Ndfa and amounts of N fixed using the two grasses. N fixation values are back transformed means.

Appendix 5. Calculation of ethylene produced with reference to a calibration of acetylene standard.

Linearity of the gas chromatography system was checked by a calibration curve of injections of known concentration of ethylene. A 1000 ppm ethylene was used to determine the slope of the curve R, where R = peak area/ethylene production. The 1000 ppm ethylene was injected periodically throughout the sample analysis as a system check. Where duplicate samples were collected the ethylene peak were averaged. The back ground level peak area of ethylene in the acetylene used was subtracted from the sample peak area. This was then divided by R, adjusted for incubation time and volume. Ethylene production was expressed as nmole C_2H_4/core/30 minutes.

This was calculated as: - ((s-b)/R/(30min/inc)x(vol.cf))

Where:-

s = mean peak area

b = mean blank peak

R = slope of calibration curve (1000 ppm peak area/1000)

Inc = incubation time (minutes)

Vol. cf = 46.43 (conversion of ppm to nmoles in jar)

The calculation from ppm to mol of ethylene

Ethylene 1000 ppm from the cylinder was used to produce the standard:

1 ppm = µL (C_2H_4 at stp)/L(air at stp)

1mol = 28g C_2H_4 (stp) = 22.4L

If 22.4L = 1mol

1L = 1x1/22.4 = 0.04464mol

0.04464mol = 1L(C_2H_4)

0.04464 micromol = 1μL

44.64 nanomol = 1μL

1 ppm = 44.64 nmol C_2H_2/L air

If vol of jar for the incubation is 1040 ml

then 1 ppm = 44.64/1000 x1040

= 46.43 nmol C_2H_4/core incubated in the 1040 ml jar.