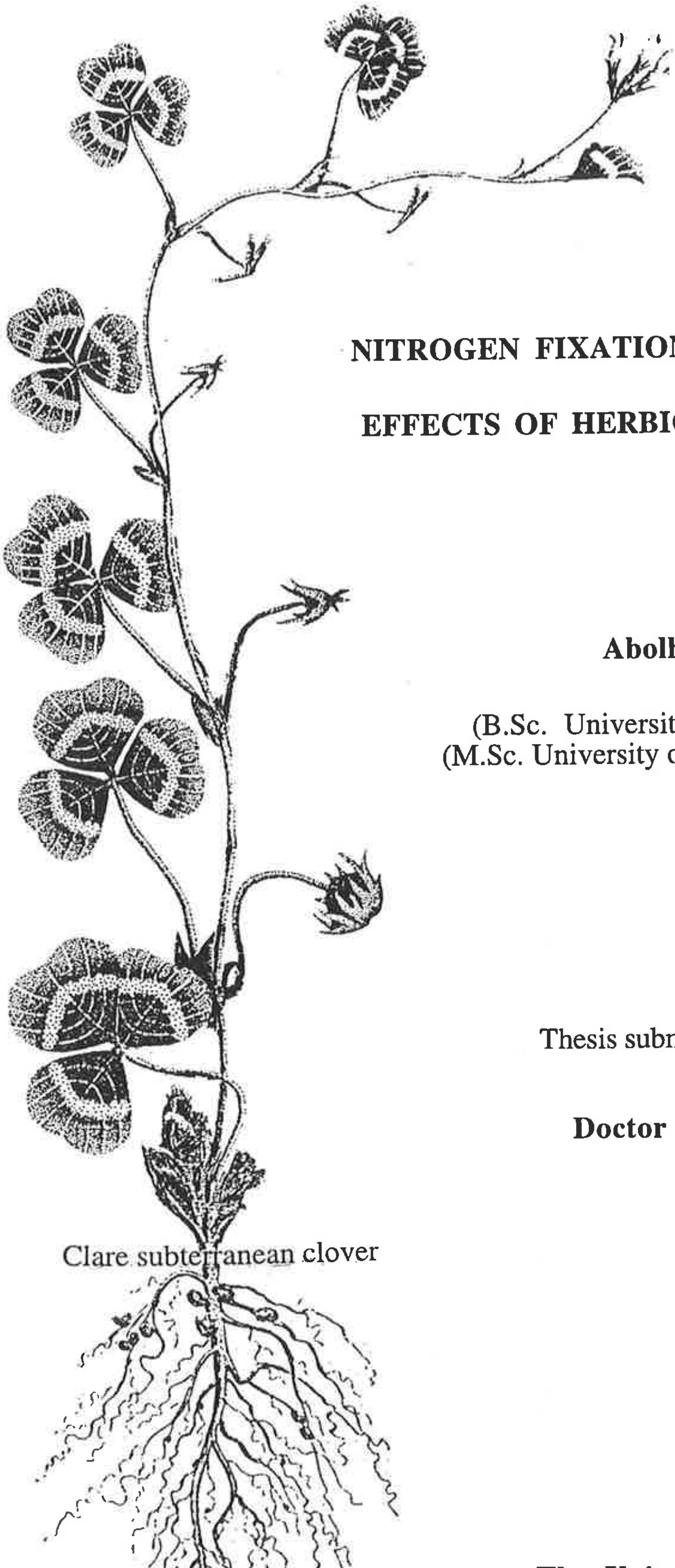

Clare subterranean clover

NITROGEN FIXATION BY PASTURE LEGUMES: EFFECTS OF HERBICIDES AND DEFOLIATION

by

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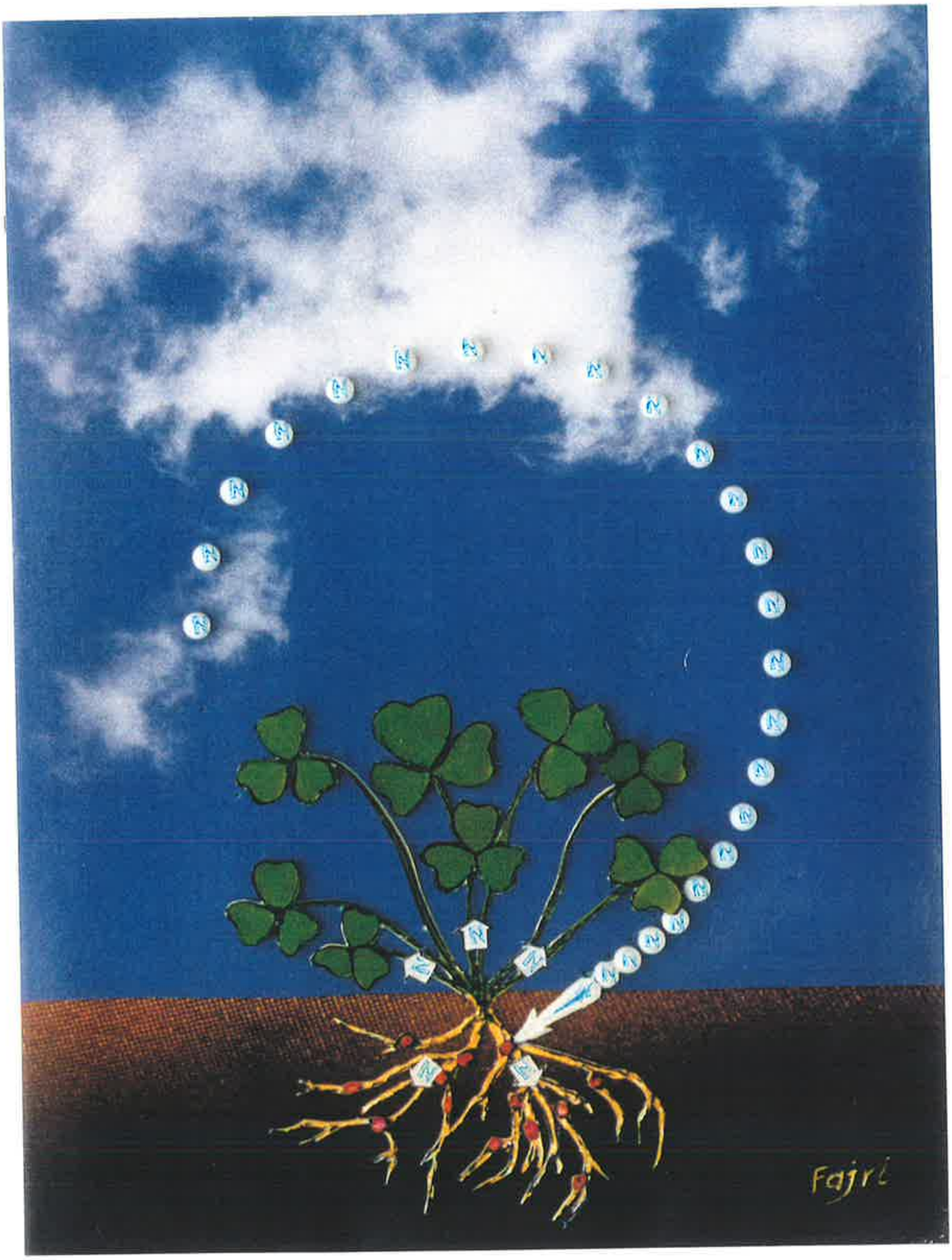

A continuous input of atmospheric nitrogen to the soil via nodulated legumes is vital for sustainable crop-pasture-livestock farming systems

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ABSTRACT

Weed control strategies such as herbicide application and/or mechanical defoliation can influence biological nitrogen fixation (BNF) processes in pasture legumes. Two research priorities are identified and studied, viz: (i) the evaluation of the effects of herbicide on growth and BNF, which was addressed in two glasshouse and two field experiments , (ii) comparing the effects of herbicide use on growth and BNF of the legumes with those of mechanical defoliation practices used as an alternative to herbicide use, which was tested in two field experiments.

(i) EVALUATION OF HERBICIDE EFFECTS ON LEGUMES.

In order to test the effects of post-emergent application of the herbicide 2,4-DB [4-(2,4-dichlorophenoxy) butric acid] on yield, nodulation and nitrogen fixation of annual medics in a pure legume sward, various medic species and cultivars were used, viz: *Medicago truncutula* cvv.Caliph, Jemalong, Mogul, Parabinga and Paraggio; *M. littoralis* cvv. Harbinger and Harbinger(AR); *M. scutellata* cvv. Kelson and Sava and *M. rugosa* cv. Paraponto. These were screened in a glasshouse experiment for response to post-emergence application of the broadleaf herbicide 2,4-DB. The medics were sprayed at 0, 1.4 and 2.8 L/ha (i.e. control, half-recommended and recommended rates) at the first and fifth trifoliate leaf stage (early and late), and the effects measured 24 days later. An in *situ* acetylene reduction (AR) assay system was used for to measure nitrogen-fixing activity in each pot. The data on the dry weight of plant shoots showed marked differences between the genotypes in their response to the herbicide, with strong evidence that effects were dependent on the time of application. For example, Harbinger (AR) was moderately affected by the early application, and severely affected by the late application, which contrasted with Paraggio, where the reverse occurred, and Kelson, which was significantly affected at both times of herbicide application. The herbicide 2,4-DB retarded nodule development severely in the upper root zone of most medics tested, especially with the early application. Higher nodule numbers occurred in the lower root zone of treated plants, which indicated some recovery of the symbiotic system. The AR assay results also showed significant differences

between unsprayed plants and plants sprayed at recommended and half-recommended rates of the herbicide at both times of application. The results indicated that the cultivars responded differently to both herbicide rate and application time in their relative nitrogenase activities. The AR activity ranged from 117-1054 µmol $C_2H_4/m^2/h$. The relationship between AR activity, nodule number and dry weight also differed markedly between species and cultivars.

Field studies to examine the effects of a range of pre-emergent herbicides on yield and nodulation of annual medics and subterranean clovers in a pure legume sward were made using five annual medics (*Medicago truncatula* cvv. Caliph, Mogul and Paraggio; *M. polymorpha* cv. Santigo; *M. scutellata* cv. Sava) and seven clover cultivars (*Trifolium subterraneum* var. subterraneum cvv. Dalkeith, Daliak, Seaton Park and Junee; *T. subterraneum* var. brachycalycinum cvv. Clare and Rosedale; *T. subterraneum* var yanninicum cv. Trikkala). A range of herbicides (mixtures containing Imazethapyr, Flumetsulam, Simazine, Metribuzin, Diuron and Metolachlor) were used at the recommended rates to determine whether there were any differences between the legume species and cultivars in susceptibility to the pre-emergent herbicides; and especially the effects of herbicides on dry weight and nodule numbers.

The smallest reduction in biomass and nodule numbers was found in Caliph and Sava treated with Simazine 1.0 L+ Imazethapyr 150 mL/ha, and the largest reduction was recorded for the same cultivars with Simazine 2.0 L/ha. A strong correlation was found between dry matter yield and nodule numbers in Mogul and Paraggio, while no such correlation was found in the cultivars Rosedale and Junee. As cultivars differ in their susceptibility to herbicides, it was concluded that extending results of herbicide application from one cultivar to an other, needs to be done with caution.

To identify the effects of time and rate of application of herbicides on the dry weight and nitrogen fixation of clover in a grassy pasture, a field study assessed the impact of Simazine/Paraquat and Simazine/Fluazifop-P at different rates (0.9+0.3 and 0.75+0.25 kg active ingredient/ha) and times (25/6, 9/7, 24/7, 6/8, and 24/8/92) and Simazine/Fluazifop-P at 0.9+0.5 kg active ingredient/ha at two times (25/6 and 9/7/92) on subterranean clover and barley grass. Legume and grass dry matter yield and fixed nitrogen alteration were used as parameters. In this experiment most herbicide treatments reduced grass components and increased clover biomass relative to the unsprayed control. There were marked differences in grass control following the use of the two rates of Simazine/Paraquat. The higher rate significantly increased roots of clover and decreased those of grass relative to the unsprayed weedy control, while the lower rate did not cause a significant difference. Also, the higher rate of the herbicide was more effective in decreasing grass shoots than the lower rate. Early application of Simazine/Paraquat was more efficient and significantly reduced the production of grass and increased that of clover, while Simazine/Fluazifop-P was more effective at depressing grass at the second time of application.

Nitrogen fixation by the legume in the grassy pasture was influenced by two factors: competition with the associated grass, and the adverse effects of the herbicide on nodulation and nitrogen fixation. In most cases, the effects of the herbicides on nitrogen fixation was more important than the effects from grass competition. The ^{15}N natural abundance technique was used to determine the amount of plant nitrogen derived from the atmosphere, using barley grass (*Hordeum leporinum*) as the non-fixing reference species. Fixed nitrogen in the root system was reduced in the legume /grass pasture by early application of the higher rate of Simazine/Paraquat, while in clover shoots there was a tendency for the fixed nitrogen to be increased by early herbicide application, which was due to increased legume shoot dry weight caused by a decrease in grass weed competition. The addition of wetting agent to the spray solution increased herbicide effectiveness.

Isotopic fractionation associated with N_2 fixation is needed for accurate estimation of the proportion (P) of the legume N due fixation. Therefore isotopic fractionation (B) which occurred during N_2 fixation by the three legumes (*Trifolium subterraneum cvv. Mt Barker* and *Clare*, and *Medicago truncutula* cv. *Paraggio*) used in the experiments was measured to adjust for the isotope fractionation in the calculation of the proportion of fixed nitrogen from N_2, using the natural ^{15}N abundance method. The isotope fractionation effect on N_2 fixation by the three legume species was determined from the change in ^{15}N abundance between atmospheric N_2 and the total N of plants grown under a controlled environment in a glasshouse with N-free nutrient solution and growth medium. The B value for Mt-Barker shoots and roots was found to be 0.48 and 0.56 respectively, in Clare shoots and roots 0.52 and 0.81 and in Paraggio shoots and roots 0.50 and 0.62.

(ii) COMPARING THE EFFECTS OF HERBICIDE WITH MECHANICAL DEFOLIATION ON THE PRODUCTION AND NITROGEN FIXATION OF LEGUMES

If mechanical defoliation is used as an alternative to herbicide in a legume-based pasture, how do these two methods affect the symbiotic N_2 fixation of the legume? Two field experiments were set up at the Waite Institute to determine the impact of herbicide the 2,4-DB on herbage production, N_2 fixation and seed yield of the annual pasture legume, Paraggio barrel medic, sown at three rates (20, 100 and 500 kg/ha) and Clare subterranean clover sown at 100 kg/ha. The effects of the herbicide were then compared with mechanical defoliation at different times: early (6 August.), early and late (6 August. and 5 September) and late (5 September).

The herbicide application reduced dry matter yield of Paraggio medic sown at 20 and 100 kg/ha, but not at 500 kg/ha and reduced seed yield at sowing rates of 100 and 500 kg/ha. The plants showed a slight reduction in fixed nitrogen from the unsprayed control. Early defoliation did not affect total cumulative dry matter yield at any of the sowing rates, and also did not change seed yields in the 20 and 100 kg/ha sowing rate swards, but significantly increased seed yield for 500 kg/ha sown swards. A single cut on 6 September (late defoliation) significantly reduced plant dry matter and seed yield at 20 kg/ha and killed all plants at 100 and 500 kg/ha sowing rates. Two defoliations (early and late) also reduced total cumulative dry matter and seed yield at all sowing rates relative to the control. An increase was observed in N_2 fixation rate following defoliation, particularly the early defoliation, but the difference was not significant compared to the control. The results indicate that the effect of defoliation on annual pasture legume production varies with sowing rates, timing and frequency of defoliation.

The relative effects of herbicide application and defoliation on plant production and N_2 fixation of two annual pasture legumes, Paraggio barrel medic and Clare subterranean clover, were also considered. The herbicide application reduced growth rate and seed yield in the both species compared with the control. The reduction in growth rate of Paraggio was apparent at the second harvest (7 October), while with Clare the reduction was apparent at the first harvest (3 September). This indicates that the herbicide application affected growth rate in Clare more rapidly than Paraggio. Early defoliation did not affect dry matter yields in either species, but two defoliations (early and late) and late defoliation reduced dry matter yield in both species compared with the control. A major difference between Clare and Paraggio was in their ability to recover after late defoliation. In Paraggio the late defoliation resulted in death of plants while Clare recovered after the late defoliation. The agronomic superiority of Clare over Paraggio in this experiment can be largely attributed to a recovery after late defoliation. In this experiment also defoliation/herbicide treatments did not significantly reduce N_2 fixation in Paraggio and Clare, but nitrogen fixation in early-defoliated legumes (in both medic and clover) was significantly higher than in legumes sprayed with the herbicide 2,4-DB.

The important finding of these two last experiments is that the early-defoliated legumes fixed significantly more atmospheric nitrogen than the herbicide-sprayed plants.

In conclusion, the studies indicate that the use of mechanical defoliation for weed control in a pasture, as an alternative to herbicide use, significantly increased N_2 fixation in both legume swards.

STATEMENT

This thesis contains no work which has been accepted for the award of degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text. I consent to the thesis being made available for photocopying and loan.

Abolhassan Fajri

PUBLICATIONS

Two papers, containing some results from this thesis, have been published in the 8th Australian Agronomy Conference, Toowoomba, Queensland, 1996.

EFFECTS OF TIME OF APPLICATION OF HERBICIDE ON THE YIELD AND NITROGEN FIXATION OF CLOVER IN GRASSY PASTURE

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EFFECTS OF A RANGE OF PRE-EMERGENT HERBICIDES ON YIELD AND NODULATION OF ANNUAL MEDICS AND SUBTERRANEAN CLOVER

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CHAPTER 1

1 GENERAL INTRODUCTION

The focus of this thesis is on biological nitrogen fixation (BNF) because this is a major beneficial feature of legume pastures. BNF can be a major source of nitrogen (N) in a symbiotic system and as such is desirable for agricultural sustainability. There have been many reviews on BNF including those of Ladd *et al.* (1983), Heichel (1987), Peoples and Craswell (1992) and Butler (1993b). Biological nitrogen fixation by nodulated legumes involves a continuous input of organic nitrogen into the soil, via the reduction of N_2 gas from the air (78% of air is N2) to ammonium ions. It is known that non-legume plants are unable to convert N_2 to ammonia, and can only use combined forms of N. So the importance of BNF in pasture legumes is to provide available combined N from the air for the maintenance or improvement of soil N levels and to ensure highly nutritious grass/legume sward production without addition of N fertiliser.

Since the discovery more than 100 years ago of the N_2 fixing capacity of the legume-rhizobial symbiosis, the processes of this system have become well established and have played an important role in raising the nitrogen status of soils. Total world biological N_2 fixation has been calculated to range from 139 to 172 x 10^6 tons per year (Burns and Hardy 1975; Paul 1988; Ishizuka 1992). According to Ishizuka (1992) the yearly quantity of biologically-fixed N is three times that of industrially-fixed N. Therefore, the nitrogen fixing system of legumes offers an economic and ecological contribution to improving internal resources and reducing external inputs (Bohlool *et al.* 1992). However, in many pastures, BNF is far from ideal and many factors can influence the system. These include weed control practices.

Weeds in pastures, particularly those weeds with a high rate of spread, are likely to be troublesome and in many situations affect the success of pasture production and weed control practices are therefore required. It has been documented that weeds in pastures can poison grazing animals, produce physical discomfort, or damage and reduce animal productivity (Combellack 1989; Carter 1990a). Weeds can also compete with the legume component in a pasture and influence nitrogen fixation and production (Butler and Ladd 1985b). Therefore, it is important to acknowledge the integral nature of weed control in pasture management. An integrated approach to weed control in pastures requires efficient herbicide use as part of on appropriate weed management strategy (Combellack 1992; Morgan 1992). Therefore, an additional focus of this study was to investigate factors which influence the efficiency of herbicide use in legume-based pastures and comparing this method of weed control with mechanical defoliation.

Much of the literature on the subject of herbicide use in pasture has concluded that there is a need for further study of the interaction of the ecology of pastures, weeds and herbicide use. For example, the use of herbicides to control weeds in legume-based pastures has a number of potential adverse effects on the yield and legume content of the pasture (Taylor *et al.* 1989; Watson 1992). Herbicides may decrease the nodulation of legumes and consequent biological nitrogen fixation (Lindstrom *et al.* 1985; Patrick *et al.* 1985; Eberbach and Douglas 1989; Pozuelo *et al.* 1989; Sandhu *et al.* 1991; Young *et al.* 1992). In addition, herbicide application to pastures affects the soil microbial biomass, nitrification, denitrification, soil respiration and enzyme activity (Carlisle and Trevors 1986 ; Helweg 1986; Yeomans and Bremner 1987; Wardle and Parkinson 1990). Continued use of the same herbicide may lead to herbicide-resistant weeds developing in pastures and herbicide of residues in pasture plants may contaminate meat or milk. However, the findings reported above seem to indicate that the advantages of herbicide use outweigh the disadvantages. One way to overcome the disadvantages may be to reduce herbicide dependence in pasture management. The first step in reducing herbicide dependence is by using non-chemical control methods whenever possible and, when it is absolutely necessary, using an efficient herbicide in an appropriate way (Morgan 1992). This may be one of the cheapest ways of removing pasture weeds, if it is properly managed (Wilson and Simpson 1993). Therefore, there is a need to consider improvement in the efficiency of herbicide use as well as in non-chemical weed management strategies including the role of mechanical defoliation and/or grazing animals (Carter 1990a).

The efficiency of herbicide use in legume-based pasture can be altered by a number of factors, including herbicide type, rate and timing of application. Many studies have clearly demonstrated the relative benefits of herbicide, when used at the minimum application rate to achieve weed control. This optimises economic returns to the user and has minimal adverse effects on the environment. Timing is critical in weed control, according to Combellack (1992); under most circumstances, late-emerging weeds are few in number and not as damaging as the weeds emerging earlier. Therefore, it is accepted that the benefits from early removal of weeds may ensure the effectiveness of a lower dose of herbicide. Three experiments reported in this thesis evaluated the effectiveness of herbicides with special reference to the following aspects: type of herbicide used; the role of herbicide mixtures; timing and rate of application and response of different species and cultivars of pasture legumes to herbicides in terms of growth, nodulation and N_2 fixation.

Mechanical defoliation in pastures is an effective and sustainable method of weed control particularly suitable for the control of woody weeds like bracken. Early methods involved cutting or hoeing the weeds: later, with mechanisation, mowing of pasture weeds at the critical time before seed maturation became popular. Cutting and defoliation affect the growth rate and persistence of pasture species as well as weeds. The challenge confronting cutting management is to maximise the persistence and productivity of desirable species while suppressing the undesirable species. Crocker and Tiver (1948) reported that mowing for pasture hay, which is commonly practised in South Australia, often increases the proportion of clover, and reduces that of grasses, in a grass-legume pasture. Also Yeatman (1992) claimed that in South Australia slashing encourages a good legume stand with some grasses, and can stop mustard and turnip from setting seed if done before flowering. However, the effectiveness of mechanical defoliation as a weed control measure in legume pastures has not been extensively investigated, and was thus an additional focus of study in this thesis.

The information derived from the experiments on herbicide effectiveness was investigated further using two field experiments at the Waite Agricultural Research Institute. These experiments compared herbicide use with that of mechanical defoliation and concerned: (i) the effects of defoliation by cutting and of herbicide application on the rate of nitrogen fixation and growth of barrel medic (at three densities) throughout the growing season, and (ii) the effects of cutting and herbicide treatment on the growth and nitrogen fixation of two different species of pasture legume (annual medic and subterranean clover). The objectives of these two final experiments were three-fold: first, to investigate the effects of defoliation and herbicide application on the productivity of Paraggio barrel medic and Clare subterranean clover; second, to measure the content of biologically-fixed nitrogen in these legumes when grown at different densities and third to compare the effects of the herbicide with that of defoliation on legume plants, in order to investigate whether defoliation has less adverse effects than herbicide use, and whether it could be used as an effective alternative method of weed control.

In brief, the various experiments detailed in this thesis were aimed at evaluating the impact of various herbicides and herbicide mixtures on the growth, nodulation and nitrogen fixation of annual pasture legumes, the efficacy of the herbicides for weed control, and the potential role of mechanical defoliation to replace herbicides for weed control in pastures leading to lower- cost and more-sustainable farming systems.

CHAPTER 2

2 LITERATURE REVIEW

2.1 ROLE OF PASTURE LEGUMES

In Australia, legume pastures occupy about 40 million (M) ha. These legume pastures are the most important feed resource for livestock (Carter 1981), the pastures increase soil fertility through fixation of atmospheric nitrogen (Butler 1986), which in turn supports the production of crops in pasture-cereal crop rotation systems (Reeves 1987). The importance of pasture legumes mainly relates to their capacity for biological nitrogen fixation (BNF). Nitrogen fixation processes are the key to maintaining or increasing soil nitrogen in the cropping system, and for providing large quantities of high-quality livestock feed in the grazing system (Robson 1990). The self-regenerating annual pasture legumes, mainly medics (*Medicago* spp.) and subterranean clover (*Trifolium subterraneum*) are the basis of successful ley-farming systems in southern Australia.

2.1.1 LEGUME-RHIZOBIAL SYMBIOSIS SYSTEMS

A general overview covering the value of legumes in soil fertility as green-manure was given by Columella nearly 2000 years ago (Ash *et al.* 1954,1960). It was about 200 years ago that the legume-rhizobial symbiosis system (biological nitrogen fixation) was recognised and the reason why legumes increase soil fertility was found. Nitrogen fixation is a most important process for maintaining or improving soil N levels of many agricultural systems. In nitrogen-deficient soils, this attribute appears to be a "godsend" particularly in a ley farming system. Many studies made in different pastures have shown a wide range of values for N fixation by legumes. According to Rowland *et al.* (1980) the total level of N accumulating under legume pastures has been estimated between 45-336 kg N/ha per year according to growth conditions. In southern Australia, the increase in soil total N varies from 9 to 350 kg N/ha per year with an average of 69 kg N/ha year. An input of 60 kgN/ha/year by subterranean clover (sub clover) pastures was measured by Kohn *et al.* (1977). Although higher rates exceeding 100 kg N/ha/year were reported by Holford (1981) and Simpson

(1987) under lucerne in irrigation systems, Simpson *et al.* (1974) showed that the increase in soil N in pasture grazing experiments by legume *Rhizobium* symbiosis in BNF was about 25 kg/ha/year. Greenland (1971) reviewed a numbers of cases which referred to the input of soil N from legumes in Australian farming systems. The range was 30 to 155 kgN/ha per year with an average of 70 kg N/ha for non-irrigated pastures. Donald (1960) reported that over a period of one year the overall contribution of N by sub clover in South Australia was one million tonnes. The great interest in legumes is not only in the increase of total N after legumes, but also the N availability to plants. Dahmane (1978) reported that soil-mineralizable N after leguminous pastures was higher than under grass pastures. The decomposition of legume residues high in N may increase the soil pH by accumulation of NH_4 and this increases mineralization of soil organic N (Barrow 1960). However, of the organic N which is returned to the soil, only part will be available to the next crop. Ladd *et al.* (1981) reported that from the medic residues in the soil, 40% of N was in an available form in the first year, and some of the remaining 60% was available in subsequent years.

Legume pasture residues help to improve soil structure (Andrew 1965; Hearne 1986), improvements in soil water-holding capacity and increase nutrient availability in the soil (Buresh and De Datta 1991). Stoneman (1973) showed an increase in water stable aggregate accumulation with the number of years of sub clover leys. Legume pasture residues also contribute to increased soil microbial activity and possibility to increased N_2 fixation (Ladha *et al.* 1989).

2.1.2 GRAZING SYSTEMS

The great importance of legume pastures in animal production is due to the high protein levels in growing legumes (Carter 1986). Generally pasture legumes supply high-quality herbage for grazing livestock with high levels of protein and the legumes also supply minerals such as magnesium and calcium (Langer 1972). In a sward with a sustainable component of legumes, the nutritional value and total yield of pasture herbage was increased relative to a pasture without a legume component (Quigley 1988). The legume component in pasture can also help slow the progressive decline in livestock carrying capacity which may appear as pasture ages (Haque and Jutzi 1985).

In Australia, pastures are one of the greatest agricultural resources (Kemp and Michalk 1993). In South Australia, annual self-regenerating legumes in ley farming systems maximise grain yields from cereals and enable higher stocking rates and faster livestock growth rates (Carter 1975, 1986). In Australia, legume pastures are established on a wide range of soils and survive over differing seasonal conditions. There are approximately 460 M ha of native and improved pastures, directly and indirectly generating income worth at least $12 billion each year (Ockwell 1990). Of the 50 M ha of crop and sown pasture lands in Australia some 40 M ha is dependent on legumes to maintain soil nitrogen levels through fixation: these legumes are worth at least $ U.S.2.5 billion/year to the Australian livestock and crop industries (Carter 1981).

Pasture legumes offer an additional attribute, that of potentially increasing grassland productivity by increasing the associated grass growth via higher soil N levels. This has been described by a number of investigators: Bryan and Velasquez (1982) reported that incorporation of legumes in pasture can raise the N content of the associated grasses. Murray and Hatch (1994) found a large proportion of the N, which originated from recently-fixed N, transferred from white clover to perennial ryegrass. Burity *et al.* (1989) estimated that 20 kg N/ha was transferred from alfalfa to grass when growing in a mixture and during regrowth between two sequential harvests. Brophy and Heichel (1989), Ta *et al.* (1989) and Tomm *et al.* (1994) also reported nitrogen transfer from alfalfa roots. Haque and Jutzi (1985) showed a rapid N transfer in mixed pasture sward, while Peoples and Craswell (1992) suggested that direct transfer of N from legume to companion grasses may occur only slowly with time. However, in mixed legume/grass pastures, the below-ground transfer of fixed N from pasture legumes to grasses (Table 2.1.) was estimated to be 2-26% of the total BNF in the legume and accounted for 8-39% of the N recovered in the grass (Ledgard and Steele 1992).

Table 2.1 Below-ground transfer of fixed N from legumes to associated grass in mixed pastures (Estimated using ^{15}N).

Legume	BNF	N transfer			Reference
	kg/ha/ year	kg/ha/ year	As % of BNF	As % of grass N	
Lucerne	93-258	3-27	7	37	Burity *et al.* (1989)
Lucerne	51-172	7-13	10	22	Ta and Faris (1987)
Lucerne	114-282	3-5	2	8	Hardarson *et al.* (1988)
White clover	83-283	11-52	21	38	Boller and Nosberger (1987)
Red clover	49-373	14-42	23	39	Boller and Nosberger (1987)
White clover	224-291	54-102	26	27	Ledgard (1991)

Source: Ledgard and Steele (1992)

2.1.3 CROPPING SYSTEMS

Symbiotic N_2 fixation by pasture legumes and input of the N to cereal-growing soils is considered to be of major importance for maintaining adequate N for the subsequent cropping system. Much has been written on these aspects of pasture-crop farming systems in Australia. According to Carter (1981, 1987) and Thorn (1989) Australia has some 50 M ha of crop and sown pasture lands. Self-regenerating medics (*Medicago* spp.) and clovers (*Trifolium* spp.) occupy some 40 M ha and each year there are some 27 M ha of pastures used for grazing and worth in excess of US $ 2,500 M to the crop and livestock industries. The other 13 M ha is used for cropping in the rotation. The winter-dominant rainfall area of southern Australia has traditionally depended upon the process of N_2 fixation by legumes to maintain soil N levels.

Naturalised annual medics and clovers in South Australia were encouraged with superphosphate following Howard's promotion of subterraneum clover (and sale of seed) in the early 20th century. Later both medics and subterranean clover cultivars were sown extensively in the cereal belt following World War II in the late 1940^{s} and 1950^{s} to maintain or improve levels of soil nitrogen for cereal crops (Carter 1975; Russell 1980). The role of pasture legumes in the N economy of these rotations has been studied. They can add approximately 118 kg/ha fixed N from sub clover and 180 kg N/ha from white clover each year, (Ladd and Butler 1992). Subterranean clover and annual medics are the most important legumes maintaining and improving soil fertility in cropping rotations throughout southern-Australia (Carter 1975). The positive residual effects of lucerne pasture on subsequent wheat yield in the northern wheat belt of Australia have been demonstrated by Holford (1981). Two and half years of grazed lucerne can increase the yields of five subsequent crops of wheat on a black earth and two crops on a red earth, where the amount of fixed N input on the two soil types was estimated to be 140 and 110 kg/ha respectively each year. The biological nitrogen fixation (BNF) will be largely related to the dry matter yield of the legume in most cases (Butler 1987). Therefore, well-managed legume pastures are well capable of contributing substantial amounts of fixed N to a soil and ultimately to a following wheat crop (Russell 1980).

Legume-based leys grown in rotation with cereal crops are also important in pest and disease control: for example, legumes are not host plants for Take-all (*Gaeumannomyces graminis* var. *tritici*) thus a legume-dominant ley is important for the control of this disease (Zogg 1969, 1972). Medics and sub clover in pastures also appear to reduce the incidence of, or damaged by cereal cyst nematodes (Barry *et al.* 1974).

2.2 FACTORS AFFECTING NITROGEN FIXATION

Biological nitrogen fixation by legumes in pastures is influenced by groups of primary factors, which can be divided into soil factors, climatic factors and management factors.

Soil factors: chemical, physical and biological.

Climatic factors: water supply, temperature and light.

Management factors: grazing, fertility, weed control, pest and disease control.

2.2.1 SOIL FACTORS

2.2.1.1 Chemical properties:

Nitrogen : In leguminous plants, during their early stage of growth, initiation of symbiotic nitrogen (N) fixation occurs rapidly, such that only a small amount of available soil and seed N are necessary to start nodulation and to help early growth. In small seeds, with a limited reserve of seed N, a "starter effect", due to a low concentration of available soil N at sowing, may be demonstrated (Richard and Soper 1979). In these circumstances, after the seed reserves are exhausted, legumes tend to go through a "nitrogen hunger" period (Pate and Layzell 1990). A demand for small amounts of combined N at germination were reported by others: in medic (Pate and Dart 1961; Dart and Wildon 1970), alfalfa (Richardson *et al.* 1957; Allos and Bartholomew 1959), subterranean clover (Bauma 1970; Silsbury 1984). Harper and Cooper (1971) and Hardy *et al.* (1980) reported that the combined N in the soil may inhibit the N_2 fixation process at the early growth stages of the legume. An effect of environmental factors such as temperature on the starter N requirement of legumes is possible (Jones *et al.* 1981). This aspect will be explained in the section on climatic factors.

Despite the promotive effects of small amounts of combined N during early stages of growth, a high level of combined N has a depressive effect on nodule initiation and development, in addition to reducing N_2 fixation of already-formed and previously-active nodules (Wagner and Zapata 1982; Streeter 1988). The soil N and N_2 fixation interaction has been studied in different pasture types. Høgh-Jensen and Schjoerring (1994) compared the effects of two levels of nitrogen application (20 and 400kg N/ha) on a mixture of white clover, red clover and ryegrass and reported that fixed nitrogen constituted between 73% and 96% of the harvested clover nitrogen at low-N, but only 50 and 64% of harvested clover nitrogen at the high-applied N level. The amount of fixed nitrogen was recorded between 31-72 kg/ha for the high-N treatment, but 118-161 kg/ha for the low-N treatment.

The important factors affecting soil N build up from legume N_2 fixation are as follows:
Legume tolerance to high soil nitrogen: Variation in symbiotic tolerance to nitrate amongst legume species and cultivars is common (Harper and Gibson 1984; Hardarson *et al.* 1984; Gibson and Harper 1985; Rys and Mytton 1985; Buttery and Dirks 1987; Herridge and Bergersen 1988; Park and Buttery 1989; Ewing and Robson 1990). In addition to effects on species, the strain of *Rhizobium* may have an effect on the degree of NO_3-inhibition (Gibson 1971; Munns 1977; Evans 1982). Manhart and Wong (1979) tested the effects of different strains of *Rhizobium* on nitrogenase activities of legumes and concluded that, when a legume which is more resistant to NO_3 inhibition is nodulated by a *Rhizobium* strain which is also more resistant to the NO_3 effect, the resulting legume-*Rhizobium* combination has additive resistance to NO_3 inhibition.

Seasonal change: In the field, soil moisture is an important environmental variable influencing the symbiotic response of legumes to nitrate in the soil solution (Streeter 1972). The level of inorganic soil N is often high during dry summer conditions since, as soil moisture decreases, nitrate in the soil solution will become more concentrated. This can lead to a decline in N_2 fixation by the legume (Ledgard *et al.* 1987). Water stress can directly result in a decrease in symbiotic N_2 fixation and growth of legumes (DeJong and Phillips 1982; Aparicio-Tejo and Sanchez-Diez 1982). Temperature can be a factor influencing the requirement for soil NO_3 (Jones *et al.* 1981). It is recognised that there are differences in sensitivity to nitrate between nodules of differing ages, mature nodule nitrogenase activity being inhibited more than younger nodule activity (Rigaud 1981).

Animal excreta : The grazing animal influences cycling of N in pasture systems: 75-95% of the N consumed by grazing animals is excreted as dung and urine. Excretion rates equivalent to up to 1200 kg N/ha have been measured in localised areas (Henzell and Ross 1973; Steele 1982). According to Barrow and Lambourne (1962), urine may contain between 45 and 80% of excreted N, being low from low-N feed (1.5%) and high from high-N content feed (3.0%). Inhibition of N fixation by high soil N in the area affected by urine can lead to a large decline (up to 90%) in the proportion of legume N fixed from atmospheric N_2 (Ball *et al.* 1979; Ledgard *et al.* 1982). An example of the extreme fluctuations in N fixation due to animal excreta was reported by Ledgard and Steele (1992). In an intensive dairy farm, assuming 40% of the area is affected by excreta, the N_2 fixation may decline by an average of about 60% and total BNF may decline by as much as 24% on an annual basis.

Pasture composition : The review by Butler (1988) indicated that grass associated with legume in mixed pasture will affect N_2 fixation in two ways, as follows:

(a) Favourably, by reducing available soil nitrogen levels (Leys 1990). This occurs due to grass utilising some of the soil nitrogen for its own growth. In this case, the presence of grass in mixed pastures acts to maintain inorganic soil N at a low level. Thus, where soil N levels would have been inhibitory to nitrogen fixation, the presence of grass leads the legume to fix more atmospheric nitrogen (Phillips and Bennell 1978).

(b) Unfavourably, by competition for nutrients, light and water. Broadbent *et al.* (1982) found a reduction in N_2 fixation by legumes in the presence of grass.

Phosphorus: Phosphorus (P) is needed for both attachment and infection by rhizobia and for the performance of the symbiotic system (Smith 1992a; Howieson *et al.* 1993). Further evidence on the quantity of P required was clearly shown in the study of Read and Mitchell (1983) that 12-16 molecules of phosphate are needed for every molecule of N_2 reduced to NH_3. Phosphorus deficiency is also an important factor limiting plant growth and additions of phosphatic fertilisers are needed in almost all areas where legumes are grown in Australia. These can produce spectacular increases in yield as shown in Table 2.2 which refers to a virgin, low-fertility sand, deficient in nitrogen, phosphorus, copper and zinc (Riceman 1948, cited by Donald and Prescott 1975).

Table 2.2 Responses to phosphorus, copper and zinc on Laffer sand on the 90-Mile Plain, South eastern South Australia.

Fertiliser	Sub clover (kg DM/ha)	Lucerne (kg DM/ha)
No fertiliser	187	62
Superphosphate†	625	937
Superphosphate + Copper	875	2000
Superphosphate + Copper+ Zinc	3250	2000

† c.10% P in the 1940's

Although short-term nutrient deficits do not lead immediately to a deterioration of nodule function, longer-term deficits do impact on legume nodulation, growth and nitrogen fixation (O'Hara *et al.* 1988a). The effects of P supply in legume pastures is a not a clear issue, because legumes can vary greatly in their tolerance to low soil P levels (Skerman *et al.* 1988). Cadisch *et al.* (1989) showed the responses of eight tropical legumes to P and K supply, with increases in nitrogen fixation ranging between 10 and 66 kg N/ha. Also culture solution studies of Millikan (1961) revealed that the naturalised cluster clover (*T. glomeratum*) may be more sensitive than sub clover to P deficiency. The growth of legumes differs in response to different rates of P supply. Asher and Loneragan (1967) have shown, from flowing solution culture studies, that the dry matter production of many species did not increase in proportion to the increase in P content of the solution. However, the relative growth rate of subterranean clover and barrel medic increased as P content of the solution increased from 0.04 to 1 µM, but higher rates had no additional effect on growth rates.

***Potassium*:** A qualitative requirement for potassium (K) has been reported for the activation of a range of enzymes, translocation of photosynthates, activity of stomates and control of ionic balance (Smith 1983). It is also suggested that K is an important element for nitrate metabolism in plants (Hansen 1994). A qualitative requirement for K has been reported for some rhizobia (Vincent 1977). Biological nitrogen fixation increased from 10 to 66 kg N/ha in eight tropical forage legumes following P and K addition (Cadish *et al.* 1989). An early study by Rossiter (1947) cited in Rossiter (1966) showed large differences between species in response to K concentration on a deep sandy soil at Perth, western

Australia. Increasing K supply resulted in a dry matter yield increase for *Trifolium subterraneum* from 187 to 316 g/m^2, whereas for *Lupinus digitatus* , dry matter yield decreased from 1027 to 874 g/m^2. Gladstones *et al.* (1964) confirmed that *Lupinus* species were more tolerant of K deficiency than was subterranean clover.

Calcium: Calcium (Ca) is essential for both nodulation and rhizobium multiplication (McCalla 1937; Norris 1956): however, some rhizobia had higher Ca requirements for growth, than others. Howieson *et al.* (1993) reported that the attachment of cells of *Rhizobium meliloti* to roots of seedling *Medicago polymorpha* L. and *M. murex* Wild, was greatly affected by additional P and Ca. However, studies by Loneragan and Dowling (1958), Norris (1959), Bergersen (1961) and Vincent (1962) indicated that rhizobia are not Ca-sensitive organisms, and can grow in culture with very low levels of Ca (16-31 μM). However, Ca deficiencies have been reported to depress nodule formation on legume roots under acid conditions in a number of temperate and tropical regions (e.g. *Medicago trunculata, M. scutellata, Trifolium repens, T. semipilosum, T. ruepellianum* (Andrew 1976), *T. subterraneum* (Loneragan and Dowling 1958; Lowther and Loneragan 1968, 1970), *M. sativa* (Munns 1970). Studies indicate the importance of high Ca concentrations to ameliorate aluminium toxicity (Rayor 1981; Munns 1986; Noble *et al.* 1986). Sartain and Kamprath (1975) found poor nodulation in soybean (*Glycine max*) by an Al-induced Ca deficiency. Munns (1970), Schubert *et al.* (1990), Aarons and Graham (1991) all reported an interaction between Ca and acidity in the effect on nodulation, and nodulation is particularly sensitive to Ca deficiency under moderately acid conditions. This may be due to Ca alleviation of aluminium toxicity, frequently experienced by plants at low pH, as reported by Shamsuddin *et al.* (1992) and Brady *et al.* (1993). Beck and Munns (1984, 1985) demonstrated a strong interaction between Ca and P in the nutrition of *R. meliloti*, with mM levels of Ca being required for growth with low levels of P.

Iron: Legumes require iron (Fe) for symbiotic N_2 fixation (Tang *et al.* 1991). *Rhizobium* and *Bradyrhizobium* are also dependent on an adequate iron supply in batch culture (O'Hara *et al.* 1988c; Lesueur *et al.* 1993). A qualitative requirement of Fe for rhizobia has been demonstrated by Thorne and Walker (1936), ranging from 2-200 μ M in batch culture. Fe concentration of 1.0 μ M in the flowing medium was tested on *B. japonicum* and *Bradyrhizobium* sp. grown in continuous culture. This concentration caused cell division and continued metabolism but the cells became longer and mis-shapen (O'Hara and Franklin, unpublished). A well-purified dinitrogenase from soybean nodules contained 28.8 g atoms of Fe for each mole of the enzyme (Israel *et al.* 1974). There is a higher Fe concentration in nodules than in any other plant tissue, and enhanced Fe uptake by roots is coupled with nodule initiation and development (Ragland and Theil 1993). Therefore the Fe requirements of plants using symbiotic nitrogen fixation is much greater than plants supplied with soil mineral nitrogen (Herridge and Holland 1993). Iron has been found to be essential for chlorophyll and leg haemoglobin synthesis (Smith 1983), and its limitation therefore results in very characteristic interveinal chlorosis on the young leaves. Fe deficiency occurs mainly in alkaline soils giving rise to "lime-induced chlorosis". Although Fe is abundant in soils (1-6%), it is poorly soluble at alkaline pH values under aerobic conditions (Anon. 1979 cited by O'Hara *et al.* 1988a). Therefore Fe uptake is reduced under these conditions. Research by a number of authors has indicated that Fe, is important in nodule development and subsequent N_2 fixation (O'Hara *et al.* 1988b, Tang, *et al.* 1992).

***Sulphur*:** This element plays major biochemical roles during legume symbiosis (Wolle *et al.* 1992). Sulphur (S) appears to be maintained in high concentrations in nodules, even when the host plant shows a deficiency of this element (O'Hara *et al.* 1988a) and nodule function or bacteroid activity is not directly diminished in nodules on sulphur-deficient host plants (O'Hara *et al* 1988b; Smart *et al.* 1984). Reduced weight and number of nodules occurs on S-deficient plants and may subsequently reduce N_2 fixation, since lack of S inhibits synthesis of S-containing amino acids and therefore, of proteins (Smith 1983). Where single superphosphate is used regularly, S deficiency is not common in pastures. Early work (Wagnon *et al.* 1958) demonstrated a sulphur response on rangeland by increasing the total herbage by 60%, initial responses were observed in clover growth (mostly *Trifolium microcephalum*). The effects of S deficiency may differ between species of pasture legume and in different environments. Hilder and Spencer (1954) recorded a greater response to S by *Medicago polymorpha* than *Medicago minima*. This could be due partly to a greater penetration of *Medicago minima* roots into the subsoil where they are capable of absorbing some of the sulphur which has been leached into the subsoil in sandy soils. Later, McKell and Wilson (1963) reported that rose clover may be less sensitive than sub clover to low levels of S in the soil.

***Molybdenum*:** Molybdenum (Mo) is normally a constituent of the nitrogenase enzyme , it is a component of this enzyme system and hence, an essential element for nitrogen fixation (O'Hara *et al.* 1988a). Israel *et al.* (1974) reported 1.3 g atoms of Mo for each g mole of enzyme from highly purified dinitrogenase of soybean. Symbiotically-dependent legumes have developed symptoms of N deficiency due to Mo deficiency (Smith 1983). Also, according to Smith (1983), Mo is required for nitrate reduction in plants. Mo deficiency in plants occurs mainly in acid soils, where there is a low availability of soil Mo (Russell 1960). Anderson and Spencer (1950 a) reported that the response of subterranean clover to Mo was greater where no fertiliser N was added and where the soil sulphur status was low. This interaction between molybdenum, nitrogen and sulphur and also soil pH is an example of the complexity of plant nutrition showing the interdependent effects of various factors on plant growth and nitrogen fixation. Rossiter (1966) suggested that the Mo requirement for nitrogen fixation may be higher in medics than in subterranean clovers.

***Cobalt*:** There is evidence that cobalt (Co) is essential for symbiotic nitrogen fixation in legumes (Ahmed and Evans 1959, 1961; Reisenauer 1960). Co is required by rhizobia for the synthesis of vitamin B_{12} (Dilworth and Bisseling 1984), and consequently for bacteroid multiplication (Riley and Dilworth 1985). Co is also needed for the cobalamin cofactor necessary for methionine synthesis in *Rhizobium meliloti* (Shinji *et al.* 1977). Field responses by nodulated legumes to Co fertilisation were reported by Powrie (1960) where 569 g/ha of cobaltous sulphate ($CoSO_4 . 7H_2O$) applied to a grey siliceous sand in South Australia improved the yield and increased the size of the nodules and their survival on subterranean clover (cv. Mt. Barker). In the same area, results from studies on the response of lucerne to 280 g/ha of cobaltous sulphate, indicated more nitrogen fixation and greater nodule weight after the Co addition (Powrie 1964). According to Hansen (1994), non-symbiotically grown legumes do not require Co for growth, while inoculated legumes relying upon N fixation for their N supply need this trace element to satisfy their N demand.

***Copper*:** Copper (Cu) is normally involved in protein synthesis in plants (Smith 1983) and is important for nitrogen fixation. Cartwright and Hallsworth (1970) found a reduction in dry matter yield of subterranean clover nodule bacteroids at low levels of Cu supply, and nodules on Cu-deficient clover developed more slowly as copper stress increased. Snowball *et al.* (1980) attributed poor N_2 fixation in subterranean clover to Cu deficiency.

***Other nutrients*:** Other trace elements have been reported to be essential for nitrogen fixation including nickel (Evans and Burris 1992). Boron and selenium play specific roles in biological nitrogen fixation of legumes (Ledgard and Steele 1992). The role of the various nutrient elements in nitrogen fixation is listed (Table 2.3).

Table 2.3 Summary of nutrients required at relatively high levels for specific stages of biological nitrogen fixation in legumes

Stages of biological nitrogen fixation	Nutrients required in relatively high concentration
Nodule initiation	Co
Nodule development	B, Ca
Nodule function	Mo, Fe, Co, Ca, Cu
Host plant growth	P, S, K, etc.

Sources: Robson 1978; O' Hara *et al.* 1988.

***Soil pH*:** Low pH in soils (soil acidity) has a major impact on the nodulation and nitrogen fixation of legumes. Nodulation in acid soils may be affected by reduced attachment of bacterial cells to roots (Howieson et al. 1993), or by other factors in acid soils affecting rhizobial growth and multiplication (Munns 1986; Glenn and Dilworth 1991).

In many cases soil acidity effects on nitrogen fixation can be attributed to low levels of available Ca, P, Mg and Mo and also Al and Mn toxicity. In a number of studies these elements were clearly shown to influence nodulation and nitrogen fixation of a range of legume species. Butler (1993a) in a paper concerning the effects of calcium carbonate on the growth and nitrogen fixation by subterranean clover on acid soils of pH 3.7 and 4.2, reported that, in general, the addition of calcium carbonate increased dry matter yields and the amount of N fixed by subterranean clover. Almendras and Bottomley (1987) reported a positive response to lime and phosphate application for nodulation of subterranean clover grown in P-deficient acid soil. Cameron and Newman (1958) suggested that the lack of success of medics to continue to maturity when growing in a red-brown earth soil pH 5.7 was linked with Mo deficiency.

The review by Smith (1983) indicated that Mn toxicity to legume may arise on some soils below pH 5 and the extra Mn in the soil reduces Fe deficiency in many legumes. Earlier, Vose and Jones (1963) reported a reduction of legume nodule number and size caused by Mn toxicity and increasing the Ca level could possibly reverse the effect. High levels of exchangeable Al in many acid soils generally adversely affects legume growth, rhizobia growth and survival, infection and nodule function. Wood *et al* (1984) reported that an adverse effect of Al toxicity on rhizobial growth was greater than its effect on root growth of white clover. Legumes dependent on biological nitrogen fixation are more sensitive to Al toxicity and decrease in growth more than plants dependent on uptake of soil N (Carvalho *et al.* 1980).

Plant species have been shown to differ markedly in their tolerance to different levels of soil pH. Little work has been done on the effect of high pH on legume persistence and nodulation. However, Tang and Robson (1993) found a reduction of nodulation in *Lupinus* species in soils only slightly above pH 6. In more highly alkaline soils, a reduction of nodule number or complete inhibition of nodulation was observed in several legume species (e.g. *Lupinus spp, Arachis hypogaea*) (Tang *et al.* 1991). Field studies of annual pasture legume species demonstrated that they varied in their response to soil pH (Robson 1969). According to an early study of Trumble and Donald (1938), barrel medic (*M. tribuloides*)† was suited to the calcareous alkaline soils, on which subterranean clover was unsuccessful. *M. minima* and *M. laciniata* were better suited than *M. polymorpha* to the soil acidity (pH < 6.5). There is some evidence that the range of soil pH where *M. truncutula* performs well is narrower than for *M. murex and M. polymorpha* (Young and Brockwell 1992).

† Synonym = *M. truncatula*

Soil salinity: The soluble salts in soils are mostly sodium, calcium and magnesium chloride, sulphate and bicarbonate ions. The high concentration of these salts in saline soils. influences the N_2 fixation process in legumes: this may occur because of osmotic removal of water from nodules, resulting in disruption of some mechanisms in the nodules (Wollenweber and Zechmeister-Boltenstern 1989; Khailova and Lar'Kova 1992; Mirza and Tarig 1993a, b). Studies with alfalfa by Khailova and Lar'Kova (1992) indicated that salinity inhibits specific nitrogenase activity. The nitrogen-fixing activity in alfalfa nodules grown under saline conditions (0.4% Na Cl) was 2 to 4 times lower than control plants grown without NaCl. Williamson (1978) and Pels (1978) reported that productivity of pasture legumes in irrigated areas of south-eastern Australia has been affected by soil salinization. Salinity has been found to influence nodulation and N_2 fixation by *Trifolium alexandrinum* (Bajpai and Gupta 1979) and by *T. subterraneum* (Hopmans *et al.* 1984). Greenway and Andrew (1962) found that burr medic (*M. polymorpha*) productivity was less affected by saline conditions than *M. minima*..

2.2.1.2 Physical properties

It is common knowledge that soil type is a major factor responsible for persistence and production of pasture legumes, and that greater legume production normally can be associated with higher symbiotic nitrogen fixation. Legumes are different in their adaptability to various soil types. *T. subterraneum* is often difficult to maintain in deep sandy soils (Gladstones 1960) while serradella (especially *Ornithopus compressus*) grown as a pasture legume is favoured by such soils (Ozanne *et al.* 1965). Medics prefer soils of medium to heavy texture with a high content of lime (Lazenby and Swain 1969). A pot experiment where lime was added to two acid soils, a sand and a loam, (Butler 1993a) showed that addition of lime had little effect on the proportion of fixed N and total N fixed by sub clover grown on the loam, while marked increases were found in the proportion of fixed N and amount of total N fixed by the sub clover in the sand. The reduced N_2 fixation by sub clover in the loam, came about because of the greater amount of inorganic nitrogen present in the loam, inhibiting nodulation shortly after germination.

Soil physical characteristics may aggravate either drying out or waterlogging effects on legume growth and persistence. In the field, on acid soils in moderately high rainfall areas, *Trifolium subterraneum* cv. Yarloop was long recognised for its adaptation to water-logged soil conditions but this cultivar has been replaced by cv. Trikkala which does not have the serious oestrogenic problem of Yarloop. Compared to other medics, *M. polymorpha* showed the greatest tolerance to poor drainage conditions, while *M. tribuloides* was common only on the black earths (Quinlivan, 1962 cited by Rossiter 1966).

2.2.1.3 Biological properties

Rhizobium and nodules: *Rhizobium* species infect the roots of leguminous plants and establish the nitrogen-fixing symbiosis. Nodule formation on the roots of leguminous species depends on the extent of the root hair infection by the soil bacteria. An interesting characteristic of this symbiosis is its specificity: *Rhizobium* spp. specific to an area can only infect specific host plant species (Roughley 1985; Long 1989). Therefore, it appears that an efficiently-fixing symbiosis in one soil may not be efficient in a different soil type, because different rhizobial species may inhabit different soil types.

Nodule formation and development on the roots of leguminous plants occurs in a series of stages including:

(a) *Favourable colonisation and survival of effective Rhizobium strains in the soil.* The review of Hansen (1994) indicates that generally the population density of rhizobia in soil is influenced by soil conditions such as soil pH, base saturation, soil texture, soil moisture, temperature, irradiance, as well as the presence of legumes. Wood *et al.* (1993) summarised the evidence that better nodulation and nitrogen fixation occurs when the strain of rhizobia is matched to the particular environmental stresses. They reported that the acid/Al tolerant strain of *Rhizobium leguminosarum* biovar *phaseoli* isolated from Kenyan soils produced more nodules and greater grain yields than a relatively acid/Al sensitive strain of *Rhizobium.* Also, strains of *Rhizobium fredii* were found to be more tolerant of salt than strains of *Bradyrhizobium japonicum.*

(b) *Infection of the host plant's roots.* Soil bacteria of rhizobia interact with growing root hairs of legumes and induce root hair curling, and penetrate the plant cell wall by partly dissolving it (Callaham and Torrey 1981; Turgeon and Bauer 1982). According to the review of Ledgard and Steele (1992), this early stage of infection can be strongly influenced by environmental conditions and by factors such as soil pH and soil nitrate. Nod metabolites, which are basic to nodule induction on legume roots, are synthesised by rhizobia and influenced by environmental factors. The excretion in *Rhizobium leguminosarum* bivar *trifolii* under environmental factors were investigated by McKay *et al.* (1993). They showed that the production, and especially the excretion of Nod metabolites, were markedly influenced by lowering the medium pH from pH, 7.0 to pH 5.0, the phosphate concentration from 1m M to 5μM KH_2 PO_4 and the incubation temperature from 28°C to 18°C. They also concluded that Nod metabolites in different rhizobia components responded differently to changes in environmental conditions.

(c) *Nodule initiation and development.* Concurrent with the formation of infection threads in legume roots, cortical cell division was initiated, finally leading to nodule formation (Sprent and Sprent 1990; Kijne 1992; Verma 1992). The infection thread releases bacteria into the cytoplasm. The bacteria multiply and differentiate into bacteroids (Newcomb 1981). The bacteroids are important to N_2 fixation. In legume roots, the formation of nodules is a complicated mechanism, controlled by environmental factors such as soil nitrogen (Carroll and Mathews 1990). Nodule growth and nodule activity is reduced by the addition of nitrate to the rooting medium of legumes (Gibson and Pagan 1977).

Mechanism of N_2 fixation: The legume nodules associated with eubacteria can be considered as optimised organs for the N_2 fixing cell organelle (Werner 1992). The enzyme complex of nitrogenase syntheses occurs in the bacteroids of legume root nodules. The enzyme nitrogenase reduces molecular nitrogen to ammonia (NH_3). Ammonia and amino-acids are the first stable products of biological N_2 fixation by micro-organisms (Hardy *et al.* 1968). The presence of oxygen, external nitrogen and ammonium are most important for nitrogenase synthesis. High oxygen levels and high concentrations of ammonium and mineral nitrogen repress synthesis of this enzyme (Hom *et al.* 1980; Adams and Chelm 1988). Oxygen has been known to inhibit N_2 fixation even in aerobic organisms such as *Azotobacter* (Bergersen 1971; Dilworth 1974), hence, efficient enzyme activity is maintained by reduced oxygen levels (Sprent 1979). Reduction in the flux of oxygen may be facilitated by a diffusion barrier in the inner nodule cortex (Hunt *et al.* 1988; Parsons and Day 1990). Leg haemoglobin which is a haemoprotein is present in comparatively high concentration in nodules (Bergersen 1980a) and maintains a rapid flux of oxygen but at low concentration (Sprent 1979). The review of Hansen (1994) indicated that oxygen levels in the nodule may also be regulated by carbon sources originating mainly from photosynthate in the leaves

Nitrogenase contains two components designated Fe protein and MoFe protein (the Mo and Fe elements are essential for nitrogenase). Both components are required for reduction of nitrogen to ammonia: this involves three steps. Firstly, reduction of Fe-protein by electron carriers (e.g. ferredoxin or flavodoxin). Secondly, the ATP-dependent transfer of electrons from Fe-protein to MoFe-protein (ATP is produced by oxidative phosphorylation in the bacteroids). Thirdly, electron and proton flow to N_2, which is reduced in steps until NH_3 is formed. The overall N_2-fixation process is usually indicated by the following general equation: $N_2 + 8e^- + 8H^+ + 16\ MgATP \rightarrow 2NH_3 + H_2 + 16\ MgADP + 16Pi$ where Pi is inorganic phosphate (Burris *et al.* 1980; Georgiadis *et al.* 1992; Rees *et al.* 1993).

Nitrogenase also catalyses the reduction of substrates other than N_2, e.g. C_2H_2, which nitrogenase can reduce by two electrons to yield C_2H_4 (Dilworth 1966; Schollhorn and Burris 1967). This reaction is commonly used to assay nitrogenase (AR) because C_2H_2 and C_2H_4 are easily separated and estimated by gas chromatography (Hardy and Knight 1967).

Nitrogenase catalysis is accompanied by hydrogen evolution (Simpson and Burris 1984). Hydrogen evolution measured by gas chromatography has also been used to estimate relative rates of N_2 fixation (Fuchsman and Hardy 1972).

Presence of compatible rhizobia and inoculation: When new legumes are introduced into a particular area, large numbers of rhizobia of appropriate strains should be present in the soil to ensure the early formation of many nodules with the ability to fix nitrogen. Failure of the legumes to thrive may be because of an inadequate supply of compatible and effective rhizobia in the soil (Harderson *et al.* 1993). The review of Peoples and Craswell (1992) has explained a number of conditions where the formation of an effective symbiosis between legume and rhizobia may not occur because of sub-optimal environmental conditions or an inadequate availability of effective rhizobia in the soil. These conditions are:

i) A lack of compatible rhizobia in the soil at the time of germination of legume seed.

ii) Where nodulation was inadequate on the same legume in the previous year.

iii) When the legume grows after a non-leguminous crop in a rotation.

iv) After land reclamation practices.

v) Unfavorable soil type, low or high pH soils, extremes of temperature, flooding, dry conditions before sowing.

Appropriate techniques are essential for successful inoculation. An early study by Donnan (1912) listed the following methods of inoculation for introducing effective rhizobia into the soil:

i) By mixing soil from areas growing well-nodulated legumes with soil lacking the desired rhizobia.

ii) Watering soil with slurry of rhizobia in water before sowing or after germination of the seed.

iii) Mixing *Rhizobium* with the seed prior to sowing.

Inoculation of seed with a peat-based inoculant containing the rhizobia prior to sowing is the most common form of inoculation (Keyser and Lie 1992). In this technique, the inoculant may be combined with lime, gypsum, or clay and applied to the seed with an attaching substance (glue, milk or sugar). Lime-coating can improve nodulation by increasing the soil pH around the seed (Lowther 1975). Obviously this technique would be valuable in acid soils.

The success of inoculation in the field, depends upon the soil environment, the population density of naturalised rhizobia, the rate of inoculation, the presence of toxic agrochemicals and operator ability (Moawad and Bohlool 1984; Alexander 1985; Bergersen *et al.* 1989; Smith 1992b).

2.2.2 CLIMATIC FACTORS

2.2.2.1 Temperature

Adverse temperature is an important limiting factor for both legumes and symbiotic N_2 fixation. However, it is generally considered that legumes dependent upon N_2-fixation are more sensitive than legumes grown on combined N (Roponen *et al.* 1970). Air temperature influences germination, emergence, early vegetative growth (Reed and Cocks 1982) and photosynthesis (Gaastra 1962) in plants and affects N_2 fixation indirectly. In contrast, soil temperature, related to rhizobial persistence and survival, was reported to have its main effects on both nodulation and nitrogen fixation (Graham 1992). The effectiveness of the soil temperature itself can be influenced by shoot temperature (Date and Ratcliff 1989) and also the strain of *Rhizobium* (Gibson 1976; Piha and Munns 1987). When *Trifolium subterraneum* was inoculated with *Rhizobium trifolii* strains NA 30 or CC17, these strains of *Rhizobium* showed sensitivity to high temperature and plants inoculated with these strains began to die when transferred to 28°C, while plants inoculated with strain TA1 showed a satisfactory growth.

Increases in initial nodulation with increasing temperatures has been reported for a variety of legumes. Gibson (1967) found 7°C the minimum temperature for nodulation of subterranean clover. In this experiment, time to initial nodulation increased as temperature declined below 22°C. Lie (1971) showed that the pea cultivar Iran was resistant to nodulation by *R. leguminosarum* at 20°C but not at 26°C.

Adverse soil temperatures may also influence the efficiency of uptake by the legume of other soil nutrients such as mineral N. Schomberg and Weaver (1992) showed that root temperature (18, 25 or 32°C) affects N nutrition of arrow leaf clover (*Trifolium vesiculosum* L. Savi) receiving starter N (0.5 and 1.0 mg N/plant). Temperature affected the N status of inoculated plants through its influence on nitrogen fixation, while the starter N uptake was not affected by temperature. Throughout the experiment, 25°C was the optimum for plant growth and N_2 fixation. The effectiveness of two strains of *Rhizobium leguminosarum* cv. *trifolii* was similar at the optimum temperature, but at 32°C, the effectiveness of RP 115-2 was less than that of strain 162 x 68. The time required for nodulation was also increased by high temperature (32°C).

2.2.2.2 Rainfall

Soil moisture has an important influence on nodulation and nitrogen fixation of legumes: either too much or too little precipitation can have a major impact on biological nitrogen fixation by legumes. It has been suggested that one reason for this response is that water stress reduces the availability of photosynthate to the nodules (Sprent 1972). Streeter (1993) suggested that when water-stress occurs the transport of water into the nodule and also the export of nitrogenous solutes may be limited. Also water stress may cause a reduction in respiration as well as problems from increasing O_2 concentration in the nodules. Water-stress inhibited photosynthesis and reduced N_2 fixation in *Medicago sativa* (Aparicio-Tejo and Sanchez-Diez 1982). However, Irigoyen *et al.* (1992a) examined the effects of water stress on nodulated alfalfa plants and reported that a reduction of photosynthate supply to the nodules was not the primary reason for water deficits reducing metabolic capacities in the nodules, but that water-stress directly influenced nodule metabolism. Pena-Cabriales and Castellanos (1993) reported that when nodules lost water in excess of 20% of their fresh weight, nodule function was permanently stopped in *Phaseolus vulgaris* L. plants.

Since rhizobia cannot move unaided through the soil, the distribution of nodules on the root system is restricted (Worrall and Roughley 1991) and as there is a direct relationship between rhizobia movement and amount of water applied (Hamdi 1974), it is possible to assume that limited water availability in soil may well affect nodulation and hence N2 fixation in nodulated legumes. Hansen *et al.* (1987) suggested that generally in most symbiotic systems N_2 fixation is more sensitive to soil water content than either leaf growth or transpiration rate. Water logging is common throughout the season and many legumes are intolerant to water logging, but the intolerance depends on the species (Katznelson 1970). Francis and Poole (1973) found that *Medicago* species were more sensitive to water logging than the sub clover used in their experiments. According to Hansen (1994) reduction in oxygen supply in water logged conditions may be a major problem in legume N_2 fixation.

2.2.2.3 Light

The energy requirement in plants directly and indirectly depends on the amount of radiation intercepted by the plants which operates photosynthesis mechanisms (Hansen 1994). Where light is intercepted by the leaves, photosynthate is produced and translocated to different parts of the plant to provide for its requirements. The requirement of legume nodules for an adequate supply of photosynthate to provide energy and carbon skeletons for nitrogen reduction and subsequent transport of reduced N has been well established (Atkins *et al.* 1978; Ryle *et al.* 1979; Reibach and Streeter 1983; Gordon et *al.* 1985). Legumes fixing N_2 as their source of reduced N require more energy per unit of N absorbed than those assimilating combined N from the soil (Mahon and Child 1979; Ryle *et al.* 1979). Since carbon substrates required for N_2 fixation are supplied by both stored carbohydrate and current photosynthate (Mederski and Streeter 1977; Schweitzer and Harper 1980), diurnal fluctuation in light flux density or other environment factors such as temperature may affect plant growth and N_2 fixation differently by influencing stored carbohydrate and current photosynthesis. The effect also differs considerably between legume species. Carran *et al.* (1982) observed a high rate of nitrogenase activity during the day and a low rate at night in *T. repens* at the prevailing field temperatures. Haystead *et al.* (1979) reported that there was no diurnal variation in acetylene reduction (AR) for *T. repens* at a constant temperature of 15 °C and a light intensity of 370 mol quanta m^{-2} S^{-1} for 12h. It appears that the nodules were not carbon-limited at night in this condition. They also showed that when *T. repens* was grown at the same photosynthetic photon flux density and day night temperatures of 20° and 16 °C respectively then the amount of AR was higher during the day, the higher temperature management was assumed to cause nitrogenase activity to be carbon-limited at night (Haystead and Sprent 1981). A reduction of AR rate after dark periods of more than 12h duration has been observed in *T. subterraneum* (Silsbury 1979) and in *T. repens* (Murphy 1981). However, Wheeler and Lawrie (1976) found the maximum AR rate occurred at night and not during the day in glasshouse-grown *P. sativum.* They explained that the lower AR rate during the day was because of supra-optimal temperature during the day and the higher level of AR at night was because of a delay in the transport of fixed carbon substrates to the nodules. Williams and Phillips (1980) found significantly different rates of apparent photosynthesis and AR rate during seedling development of *Glycine max* under two irradiances, the photosynthate and AR rate were lower at an irradiance of 1500 microeinsteins than in the seedlings exposed at 700 microeinsteins per meter^{-2} per second, for several weeks. Plant growth and rates of N_2 fixation became similar regardless of the irradiance after five weeks. They suggested that during plant growth, physiological adaptations of shoots to the two light levels, as well as photosynthesis *per se* affected root nodule activity.

2.2.3 MANAGEMENT PRACTICES

2.2.3.1 Grazing and clipping

Since grazing, 'topping' (i.e. mechanical defoliation with cut material left *in situ*) and silage/hay cutting are such important parts of pasture management, it is necessary to review their effects on the legume symbiotic system in pastures. The effects vary with species and amount of forage present, frequency and severity of defoliation and the environmental conditions (Gibson 1977). Defoliating shoots of legumes removes a primary source of photosynthate necessary for nodule respiration, structure and function (Butler *et al.* 1959), and for initiation and development of new nodules, and also energy for maintaining N_2 fixation (Moustafa *et al.* 1969). Understanding the effects of defoliation will depend on the defoliation practice. Ryle *et al.* (1985a) showed that the immediate effects of a single defoliation in white clover reduced the rate of N_2 fixation by more than 70%, the rate of photosynthesis by 83-96 %, and the rate of plant respiration by 30-40%. The rate of recovery of N_2 fixation suggested that this was related to the re-establishment of the plant photosynthetic capacity. Overall, up to 40% of daily photosynthate in nodulated legumes is used by the nodules (Gordon *et al.* 1987) and any factor which reduces photosynthetic products may cause a reduction in nodule respiration and N_2 fixation (Ryle *et al* . 1985a). It has been suggested that this effect is related to the high dependence of N_2 fixation on the contribution of currently-fixed carbon (Kouchi and Higuchi 1988; Hansen *et al.* 1992a,b). According to Ryle *et al.* (1979) and Haystead *et al.* (1980) plants fixing their own N_2 generally suffer higher whole-plant respiratory losses when defoliated, than those supplied with combined nitrogen: this may be a reason why plants fixing their own nitrogen have lower growth rate than those utilising combined N (Hart 1987).

Severe defoliation of white clover caused degradation of leghaemoglobin, decay and loss of nodules and consequently a reduction in nodule weight per plant (Chu and Robertson 1974). It has been reported that defoliation treatment causes nodule colour changes from pink to green or brown due to the breakdown of leghaemoglobin (Wilson 1942). Because of the close relationship between leghaemoglobin content of leguminous root nodules and N_2 fixation (Virtanen *et al.* 1947), such a change in colouration probably indicates reduced N_2 fixation. However, other reports indicate defoliation had little or no effect on legume N_2 fixation (Davidson *et al.* 1970). Different cutting frequencies have not shown any effects on N_2 fixation in white and red clover and ryegrass swards (Høgh-Jensen and Schjoerring 1994). An increase in N_2 fixation may occur following light defoliation (20% foliage removed every two weeks) when compared with uncut controls (Young *et al* . 1962).

Generally, the amount of foliage in the total herbage mass and the proportion of leaf area removed by defoliation and the amount remaining following defoliation give rise to the response of plants to defoliation (Brougham 1956; Davidson and Donald 1958). Further evidence on the interaction between defoliation and the nitrogen nutrition of legumes is provided by the studies of Chapman *et al.* (1992) who compared translocation patterns of carbon in nitrate-fed and nitrate-deficient white clover seedlings following defoliation and found a large reduction in total export of carbohydrates in N-deficient white clovers, following a severe defoliation, whereas a small increase in total export of carbohydrate in N-sufficient plants (either receiving nitrate-N or fixing their own N_2).

Under field grazing systems, in addition to decreased net photosynthetic rate and N_2 fixation by reduction of foliage (in particular leaf area) under the critical value, it seems that there are other factors affecting N_2 fixation of pasture legumes, such as the formation of inorganic N and its redistribution, via urine and faeces (Hilder 1964; Denmead *et al.* 1976). According to French (1981) the amount of N exported by a sheep is estimated to be 0.8-2 kg N each year.

Whiteman and Lulham (1970) compared cutting practice and grazing management of tropical pasture legumes and concluded that grazing caused a greater reduction than cutting on nodule weight and legume yield. Grazing management has the ability to alter botanical composition in a pasture (Carter 1986,1990a). The effects of grazing animals on individual plants in a pasture will vary with stocking rate, amount of forage present and plant growth rate (Hodgson 1966; Morris 1969). In annual pastures, heavy grazing removes a higher percentage of photosynthetic material and encourages prostrate plants e.g. pasture legumes and flat weeds, whereas light grazing (also referred to as lax grazing) encourages taller-growing species including grasses and tall weeds. In a mixed white clover pasture under two grazing intensities (especially frequent defoliation during spring), Refi *et al.* (1989) reported an annual increase of 33% in N_2 fixation and in New Zealand, Brock *et al.* (1983) observed an increase of 10% in N_2 fixation following defoliation. Grazing animals can also influence the ratio between legumes and grasses. Curll (1982) reported that in a white clover, sub clover and grass pasture, clover was less tolerant of grazing than grass.

In New Zealand permanent pastures, Clark *et al.* (1982) and Radcliffe *et al.* (1991) found large differences between goats and sheep in selection for clover during grazing. Sheep grazing led to grass-dominant pasture with a decline in clover, whereas goats selectively grazed grass and weeds and this favoured the clover. Goold (1981) reported that pasture grazed by cattle also tends to have higher clover content than pasture grazed by sheep. Sheath and Hodgson (1989) reported that increases of pasture legume persistence, under grazing by different animals, is generally greater for goats than cattle and cattle than sheep, whereas Wilson and Simpson (1993) say there is no general rule, on selection of clover, because of variable palatability of grass companion species. In annual pastures severe grazing pressure by sheep leads to dominance of small-seeded and hard-seeded legumes (e.g.*Trifolium glomeratum*) as this small seed escapes damage by chewing and the hard seed survives passage through the gut (Carter 1966; Carter *et al.* 1989; Squella 1992).

Defoliation by cutting in annual medic (*Medicago littoralis*) grown alone or with ryegrass was studied in pots and the field by Butler (1986) who reported that the response of medic to clipping differed between glasshouse and field experiments. In the glasshouse (controlled environment) clipping severely reduced N_2 fixation by medic plants, whether grown alone or with ryegrass. In the field, the clipping rarely affected N_2 fixation: however, there was a linear relationship between medic yields and fixed N in both pot and field experiments, but in the field other factors such as water limitation, rather than clipping, tended to control yield and therefore the amount of N_2 fixation.

2.2.3.2 Fertility management

In a legume pasture system soil factors such as nutrients determine productivity. A key to continued high legume production in pastures is to ensure sufficient concentration of all nutrients. Soil analysis data collected in south-eastern Australia showed that deficiencies of P, K and S were widespread throughout a sampled area (Brown *et al.* 1992). Surveys of pasture composition in south-west Victoria also reported soil P, K and S deficiencies (Quigley *et al.* 1990). Surveys of N_2 fixation processes in south-west Australia also indicated that N_2 fixation by clover was often well below expectations. The proportion of N_2 fixation from the atmosphere ranged from 18-98% (132 sites 1989) and averaged only 61% (Sanford *et al.* 1993). Evans (1992) summarised the probable causes of the decline in pasture quality over the last 20 years in south-western New South Wales. The decline in pasture quality was associated with P deficiency (especially on high Al soil with high P sorption), soil acidity (with its Al and Mn toxicities and Ca and Mo deficiencies), low seed reserves and root-rot diseases. Acidity and Al toxicity particularly affect symbiotic N_2 fixation, reducing legume yield and N content. Therefore nutrient fertility is important in determining both productivity and quality of pastures. During pasture establishment, phosphorus dressings are recommended either as small annual applications or as a large dressing less frequently. Like phosphorus, other nutrients are essential in maintaining productivity and attention should be given to the other nutrients to support productivity (Pinkerton and Randall 1993). Legumes with small seed size usually produce low seedling weights and nodule numbers in the absence of small amounts of mineral N during the early stages of growth: therefore, applications of small amounts of N fertiliser may be beneficial to the early growth of legumes (Schomberg and Weaver 1990).

Changed ecology of pastures growing in acid soils is producing changes in pasture composition and the failure of clover in permanent pasture (Cregan *et al.* 1989). Liming improves the nodulation of many temperate legumes grown in acid soils (Munns *et al.* 1977). In many acid soils, it is clear that incorporation of lime will increase availability of nutrients to pasture plants, which may affect the yield and botanical composition of the pasture and hence the level of symbiotic N_2 fixation (if increased availability of inorganic soil N does not inhibit N_2 fixation). Butler (1993a) found that lime, additional to that which maximised growth of subterranean clover grown in acid soil (sand), dramatically increased both the proportion of fixed N and the amount of nitrogen fixed. Peoples *et al.* (1995) examined the effects of surface application of lime and superphosphate to acid soils on the growth and N_2 fixation by subterranean clover in mixed pasture swards, and reported that the total pasture N was increased from 53 to 115 kg N/ha by lime applications, and the proportion of clover N derived from N_2 fixation was high (90-97%). There was an increase in clover N yield from less than 25 kg N/ha to more than 110 kg N/ha.

2.2.3.3 Pests and diseases

There are a number of serious pests and diseases which cause lack of persistence and yield of pasture legumes. In South Australia, the most important insect known to attack legume nodules is sitona weevil (*Sitona humeralis*). While legume nodules are damaged by the larvae, the adults attack the leaves of medics (Moulden 1973). Yield losses of about 50% in the winter availability of medic herbage is possible when conditions are suitable for attacks by sitona weevil, red-legged earthmite (*Halotydeus destructor*) and lucerne flea (*Sminthurus viridis*) (Matz 1973). The effects of spotted alfalfa aphids and bluegreen aphids on lucerne were reported by Lehane (1982). In northern Australia, anthracnose diseases are threatening *Stylosanthes humilis* and *S. guianensis* legumes in sown pastures (Irwin 1989). It has been reported that effects of insects or disease may result in losses of pasture plants up to 50% (subterranean clover) when the conditions are suitable for insect invasion or high disease incidence (Allen 1987; Taylor and Greenhalgh 1987; Hochman *et al.* 1990). In New Zealand, East and Pottinger (1984) estimated that up to 40% loss of pasture plants could be caused by insect pests. In white clover and alfalfa, cyst and root-knot nematodes have been reported to reduce nodulation and also attack nodules in alfalfa (Taka and Raski 1969).

2.2.3.4 Weeds

Weeds in pastures usually fit in the category of low value grasses, nitrophilous forbs, poisonous plants and unpalatable species (Combellack 1989). The proportion of weeds growing with legumes in a pasture may affect N_2 fixation in two ways: a) by occupying space that could be used by legumes, thus loss of possible N_2 production by legumes due to the space being occupied by the weed. McIvor and Smith (1973) found that in many pastures, spaced plants of capeweed (*Arctotheca calendula)* were larger than subterranean clover or annual ryegrass plants. According to Philips and Bennett (1987) pasture availability may increase when some clover plants are replaced with the same number of grass plants. b) In competition with a N_2-fixing legume. Since interference and competition between legumes and grass weeds are of great importance in pasture production, examples in this review are taken from grass/legume competition studies. Legumes generally compete with grasses for some main environment factors such as light and temperature , nutrients and water (Haynes 1980), but the trends in competition depend on the degree of defoliation. In grass-legume swards containing prostrate annual legumes like medics and subterranean clover, grazing favours legumes because a lesser proportion of photosynthetic tissue is removed than with the taller grasses. Thus, with lax grazing, grasses will dominate over legumes (Carter 1977, 1990b).

Competition for light in a grass-legume sward involved interactions with other environmental factors, for example the amount of mineral N in soil. Stern and Donald (1962) found that in a grass-clover sward increasing rates of nitrogen application increased yields of associated grass. The increased grass growth reduced light intensity at the clover leaf canopy, causing reduced growth of the clover. In a grass-legume pasture, competition for light may increase the amount of nitrogen transfer from legume to grass. This may be caused by death of nodule tissue due to the restricted supply of carbohydrates to the root system under shade conditions (Chestnutt and Lowe 1970).

Competition for nutrients appears to be more important than competition for light. In association with grasses, most legumes generally compete poorly for N. In many cases, grasses absorb more soil N than legumes, and reduce the inhibitory effect of soil N on N_2 fixation. Ledgard *et al.* (1985d) reported that, although total N yield declined by 15%, fixed N was 20% greater in subterranean clover when grown with annual ryegrass, than when grown with a slow-growing, less N-demanding phalaris. According to Butler and Ladd (1985b) although the proportion of legume N from fixation may be increased by the associated grass species, the total legume N yield is decreased through decreasing legume dry matter. Poor competition of legumes with grasses was also found in white clover for P (Jackman and Mouat 1972 a,b) and K (Mouat and Walker 1959) and for S (Neller 1960). The effects of drought on the competitive ability of perennial ryegrass and white clover were reported by Thomas (1984). Under glasshouse conditions, in previously-droughted mixtures of grass and clover, clover regrowth was less suppressed by grass than in a continuously-watered mixture. In practice, it seems that the responses to light, nutrients and moisture stress of legumes in mixed pastures, are modified by management practices applied to the pasture such as frequent defoliation and/or cutting height.

2.2.3.5 Agri-chemicals

There are available a wide range of chemical products including herbicides, insecticides and fungicides capable of controlling weeds, pests and diseases in legume pastures. These are supplied mainly as seed coatings, or as pre-emergent and or post-emergent top spraying applications. These chemicals may impact on the inoculant rhizobia, or soil rhizobia, or they may affect nodule initiation and development (Gibson 1977). Some chemicals used do not have any effect on the bacteria and symbiotic N_2 fixation by legumes, while others exert a short - or long-term inhibitory effect on the bacteria and/or symbiotic N_2 fixation, this effect being dependent on the rate of application and the inherent sensitivity of the rhizobia strains. In a study by Eberbach and Douglas (1989), the detrimental effects of nine herbicides on the growth of *Rhizobium trifolii* TA1 in liquid medium were dependant on the herbicide type. In the same study the growth of the rhizobia was significantly reduced by all concentrations of diquat, 2mg ai/L of paraquat, 10mg ai/L of glyphosate and 2mg ai/L of chlorsulfuron, while remaining herbicides. namely 2, 4-D, amitole, atrazine and diclofop-methyl and trifluralin did not suppress rhizobial growth. Many herbicides may affect nodule formation and function by influencing root development or chlorophyll synthesis. Martensson and Nilsson (1989) investigated the effects of chlorsulfuron (a broadleaf weed herbicide) on infection, nodulation, and growth of alfalfa and red clover and symbiotic performance of legume bacteria. They concluded that the legume bacteria in pure culture were unaffected by chlorsulfuron, but normal root hair infection and nodule functioning was inhibited by the herbicide. In studies on the nodulation and nitrogen fixation of red clover, using a herbicide dinoseb, it was concluded that dinoseb did not act directly on the nodules, but affected nitrogen fixation by damaging the photosynthetic parts of the plant (Lindstrom *et al.* 1985). In other studies, foliar application of bentazone and MCPA (4-chloro-2-methy-phenoxyacetic acid) at recommended rates on red clover, altered the morphology of the root hairs and reduced the nodule numbers and level of nitrogenase activity (AR) up to 34 days after herbicide application, compared with controls (Ljunggren and Martensson 1980). Herbicide at normal application rates may have different effects on the symbiotic N_2 fixation of different legumes. In a study 2, 4-DB severely inhibited the nodulation of *Lotus corniculatus* (Garcia and Jordan 1969) but had no effect on nodulation and yield of alfalfa (de Oliver *et al.* 1970).

The use of insecticides provides control of many insects in pastures, although they have also been involved in the death of inoculant rhizobia (Gibson 1977). According to Hansen (1994) direct application of pesticides to rhizobia may have more adverse effects on rhizobia than indirectly on plants. Steele *et al.* (1985) used insecticide treatments to control invertebrates on white clover/ryegrass temperate pastures, there were increases in total pasture production up to 13%, clover production up to 28% and N_2 fixation up to 57%. In this experiment the use of insecticide appeared to provide good control of soil nematodes. Other studies also reported using pesticides to control insects in subterranean clover

(Braithwait *et al.* 1958), in *Trifolium alexandrinum* (Gawaad *et al.* 1972) and in alfalfa (Russell and Coaldrake 1966) without damaging effects on the legume.

Several workers have reported the effects of fungicide treatment on nodule development, in subterranean clover (Cass Smith and Holland 1958), *Medicago truncatula* (Kleinig 1965) in alfalfa (MacKenzie *et al.* 1972). A study of the effect of fungicides on survival of *Rhizobium* on common bean seed indicated that seed treatment with Benlate together with seed inoculation reduced the amount of inoculated *Rhizobium leguminosarum* in nodules, while the same fungicide when applied in the seed furrow did not influence the survival of the inoculated strain (Romos and Ribeiro 1993).

2.2.3.6 Side effects of Agri-chemicals

It is well known that the mismanagement of many agri-chemicals, including herbicides, insecticides and fungicides in pastures can cause significant environmental problems. Efforts have been made to review the distribution of toxic substances in the environment (Combellack 1989). In his review of loss of herbicide from ground sprayers, it appears that significantly more spray loss occurred within rather than outside the target area (Combellack 1982). For example, it has been estimated that only 30% of paraquat was collected by grass weeds when sprayed for controlling grass weeds (Graham-Bryce, 1977 cited by Combellack, 1989). Herbicides have been of specific interest due to their detection in some surface and ground water sources. Surveys by Smith *et al.* (1986) and Lym and Messersmith (1988) reported residues of the herbicide picloram in underground water, although the herbicide concentration in water was significantly below that suggested as acceptable for human consumption. In another study the herbicide atrazine has been detected in ground water (Pionke *et al.* 1988). The side effects of herbicides may occur from residues in the air. The possibility of spray, as droplet drift from ground sprayers, moving up to 50 metres outside the target area in some conditions (for example windy conditions) is suggested by Combellack (1989). In the study reported by Grover *et al.* (1976) up to 30% losses of herbicide 2,4-D as a vapour, occurred. Robinson and Fox (1978) reported that

2,4-D vapour was found to spread for 16 to 90 kilometres. And again, Grover *et al* (1985) claimed that vapour loss of 2,4-D can occur following rain for up to 5 days after spraying.

Soil micro organisms may be affected when agri-chemicals are applied in agricultural systems. Effects on a microbially-mediated system may occur: for example nitrification, denitrification, soil respiration, and soil enzyme activity (Yeomans and Brenner 1987; Helweg 1986). Effects on soil algae by linuron and diuron have also been reported (Maier-Bode and Hartel 1981).

Side effects of agri-chemicals are now under investigation because of increasing numbers of pesticide-resistant species of insects and weeds (Georghiou 1986). In Australia, resistance has been recorded for barley grass to paraquat (Warner and Mackie 1983) and for Wimmera ryegrass and wild oats to diclofop methyl (Howat 1987). Barley grass also shows resistance to the herbicide diquat (Warner and Mackie 1983; Powles 1986).

2.2.3.7 Strategies to reduce agri-chemical use

Integrated weed, pest and disease management in a pasture combines the use of efficient agri-chemicals with non-chemical control techniques. The various ways to achieve efficient utilisation of agri-chemicals have been discussed by Combellack (1992). It was concluded that application of the chemical at a critical time and minimum rate to achieve a level of weed or pest control provides economic returns and minimal adverse effects on the environment. Under most circumstances in pastures, early treatment for weed removal produces both efficiency and economy. In a pasture containing subterranean clover and *Vulpia* species, mid-winter applications of paraquat were more effective in increasing clover content, than those made in spring (Barrett *et al.* 1973).

Greater than optimal dose rate may increase side effects and lower doses may reduce the chemical efficiency: Combellack (1992) claimed that industry should accept a more responsible stance to ensure the recommended dose rates of chemicals are used. There are a number of other factors which can influence agri-chemical efficiency and activity such as use of more-effective formulations and mixing with appropriate wetting agents, calibration of application equipment and favourable weather conditions (Combellack 1989, 1992).

2.2.3.8 Alternative control strategies

It is well documented that to enable reductions in agri-chemical inputs for pasture management it is necessary to integrate the various control options, including grazing, mechanical and biological control. Grazing animals will influence competition between legumes, grasses and weeds in pastures. The effects of grazing animals differ for different pasture situations. In annual and perennial grass/legume pastures a moderately high stocking density usually increases the legume component (Rossiter 1966; Carter 1977, 1985, 1990a). However, under-stocking usually leads to grass-dominant pastures with a decline in annual legumes due to competition from grasses. This trend in competition relates mainly to the removal of photosynthetic parts of the pasture plants. Clearly, the taller-growing grasses and taller edible weeds are penalised more by hard grazing as this removes a high percentage of photosynthetic material.

The impact of sheep and goats on botanical composition can be put to good use in encouraging legumes as heavy grazing favours more prostrate plants such as subterranean clover and medics (Carter 1990a).

The vital effects of the grazing animal on pasture are: defoliation, treading, recycling of nutrients and spread of seed: these effects may lead to weed distribution or weed control in pastures (Carter 1990a). Treading damage to pasture can be caused by both sheep and cattle (Edmond 1970). It was reported by Edmond (1963) that in both winter and summer, white clover was more affected by treading than perennial ryegrass leading to a grass dominant pasture. Carter and Sivalingam (1977) reported that various combinations of treading intensity (0, 12.5, 25 and 50 sheep equivalents per hectare) and treading frequency (0, 7, 14, and 28 days) caused significant reduction in establishment and productivity of subterranean clover and annual ryegrass, and a mixture of these two annual pasture species, grown on a red brown earth soil at the Waite Agricultural Research Institute. However, the Waite Institute studies on the impact of treading on annual species produced similar results to the New Zealand studies on perennial species. In both cases the grass was less affected by treading than the clover and the greater resistance of the grass to treading was ascribed to increased tensile strength of the grass leaves. This is another factor involved in the competition between legumes and weeds. Grazing by livestock may have specific effects on the plant components of a pasture as some species are palatable to sheep, others to goats, others to deer, etc.

Legumes may sometimes be dominated by grasses in a mixed pasture due to selective grazing of legumes by livestock (Watkin and Clements 1974). However, it is obvious that, weed control in pastures must be attended by management practices based on knowledge of the ecology of the weed and the pasture .

Mowing for pasture hay, often increases the proportion of clover, and reduces that of grasses (Rossiter 1966). However, the trend in competition is greatly influenced by the timing and height of mowing. If cut too late and /or too low the regrowth and seed yields are greatly reduced, leading to invasion by weeds. Capeweed content may increase in pastures following mowing in successive years (Crocker and Tiver 1948). There is a delicate balance between the production and consumption of seed of useful annual species and the impact of nitrogen fixation which may encourage invasion by non-legumes.

Mowing or cutting is a rapid and complete process which is not very representative of the pasture situation where the animals are grazing. However, mechanical defoliation can have real benefits in terms of weed control but more information is needed on the effects of defoliation of annual legume-grass pastures and consequent weed control.

In addition to the various methods of controlling botanical composition of pastures, considerable attention is being aimed at exploiting genotypic variation among crop and pasture plants as a means of reducing dependence on agri-chemicals. Legumes resistant to a number of chemicals are being selected or bred. For example, red clover has been known to be susceptible to 2,4-D (Taylor *et al.* 1982, 1987). Henderson and Claydon (1983) evaluated tolerance to the herbicide 2,4-D in red clover and observed that a significant variation in tolerance existed between cultivars. Swanson and Tomas (1979, 1983) found a callus of birdsfoot trefoil that was able to withstand comparatively high levels of 2,4-D.

Breeding legumes resistant to pests and diseases is also a method of reducing agri-chemical use: this has been reviewed by Ledgard and Steele(1992). Cultivars of lucerne can be found with resistance to many pests and diseases (Watson *et al.* 1989) although the cause of root rot in subterranean clover and resistance to *Phytophthora clandestina* remain elusive (Flett 1991).

2.3 TECHNIQUES FOR ESTIMATING NITROGEN FIXATION

There are currently two broad methodologies for measuring N_2 fixation - the direct and indirect methods. Direct methods analyse nitrogen itself to obtain the amount of N_2 fixation. Indirect methods depend on the measurement of products of the fixation reaction or closely related reactions.

2.3.1 DIRECT METHODS

2.3.1.1 ^{15}N-Isotopic techniques

Principle behind the method : Following the early study of Burris *et al.* (1942) and a number of other investigators (see review of Chalk 1985), the ^{15}N isotope dilution technique has been widely used to estimate the proportion of N in plant material derived from the atmosphere. This method is basically ideal for time integrated measurements of N_2 fixation. From two stable isotopes of nitrogen ^{15}N and ^{14}N (Peoples *et al.* 1989), the ^{15}N labelled dinitrogen is used in studies of N_2 fixation by legumes. This isotope occurs naturally in atmospheric N at an abundance of 0.3663 ± 0.0004 atoms % (Junk and Svec 1958; Mariotti 1983) and the ^{14}N makes up the remaining 99.6337% of N. The ^{15}N concentration in plant-available soil N is naturally higher than that of the atmospheric N_2 in many situations (Shearer *et al.*;1978, Karamanson *et al.* 1981; Steele *et al.* 1981). Thus it is possible to use this difference in the natural abundance of ^{15}N between soil-derived N and atmospheric N_2 to estimate of the proportion of legume N fixed from the atmosphere (Amarger *et al.* 1979; Bergersen and Turner 1983). The abundance of ^{15}N in plant-available soil N is commonly obtained by analysis of a non-fixing reference plant growing in that soil (Figure 2.1).

With increasing atmospheric N_2 fixation, the nitrogen from the atmosphere will lead to a dilution of the amount of ^{15}N present in the N_2 fixing plant, relative to a non-N_2 fixing plant. The amount of legume N derived from the atmosphere can then be calculated from the simple algebraic expressions (Peoples *et al.* 1989) (See ^{15}N analyses). The estimation of the proportion of legume N derived from N_2 fixation is independent of legume yield. However, the determination of the amount of N fixed, requires the amount of dry matter and its N content.

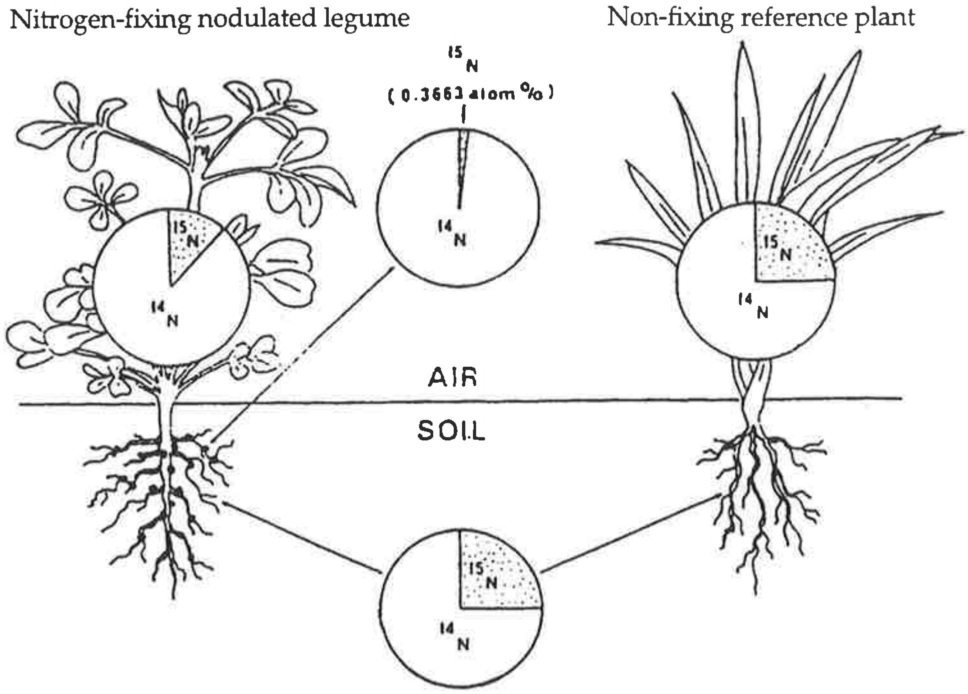


Figure 2.1 **"Diagrammatic representation of the principles involved in the ^{15}N dilution technique. Dilution of soil N by atmospheric N_2 results in a lower ^{15}N composition in the legume's production of growth than measured in the reference crop and is represented as a reduced area in the ^{15}N sector of the leguminous plant". After Peoples *et al.* (1989).**

The reference plant, should ideally exhibit the growth pattern of the legume in question, but be totally dependent on soil N for its growth (Chaiwanakupt *et al.* 1991). The main assumption inherent in the method is that the isotopic composition ($^{15}N/^{14}N$) of the N assimilated by the non-fixing reference plant and the legume from soil sources should be the same when integrated over the measurement period (Hamilton *et al.* 1991; Peoples *et al.* 1991). There may be simultaneously differences in the isotope composition of plant-available N with soil depth (Edmeades and Goh 1979). Therefore, it is important to match root distribution of the legume and reference plant at all times during growth, (Witty 1983a, b). This is particularly so in the ^{15}N-enrichment method, because ^{15}N-labelled materials are commonly applied to the soil surface (Knowles 1980) and therefore not evenly distributed down the soil profile. There is an associated decline in the $^{15}N/^{14}N$ ratio in plant-available soil N, with time and because of the release of soluble ^{14}N-compounds by mineralisation of soil organic matter. Non-nodulated legumes are reported to be suitable reference plants for the nodulated legumes of the same species (Vose *et al.* 1982). In a mixed pasture, when legumes are grown in association with grasses, selecting the associated grass as the reference plant may incur a potential error in estimation of the proportion of N_2 fixation by the legume, because of possibility of direct transfer of fixed N from legume to reference plant (associated grass), and/or the fixed legume N is transferred to the soil and subsequently taken up by the reference plant (Ledgard and Steele 1992). Any N_2 fixation by the reference plant (Bell and Nutman 1971), will also lead to the underestimation of N fixation by the legume.

^{15}N-enrichment : This procedure involves the addition of small amounts of ^{15}N-labelled fertiliser to the soil prior to the growth of the legume and reference plant. (Chalk 1985).

A problem connected with the ^{15}N-enrichment method is that the addition of ^{15}N-labelled material may reduce the fixation of N_2 by the legume. Therefore, to avoid such effects on N_2 fixation, low levels of N application (e.g.<5 kg N/ha) are preferable (Peoples *et al.* 1989). It has been reported that the rate of supply of ^{15}N-labelled fertiliser in the pasture and field studies has varied from <1 kg/ha (Goh *et al.* 1978) to 14 kg N/ha (Heichel *et al.* 1981). The selection of a non-N_2 fixing reference plant is the most important factor affecting the estimate of the proportion of N fix from N_2 fixation by this method. A number of investigators have reported that the measured amount of N_2 fixed depends on the reference plant used (e.g.Wagner and Zapata 1982; Witty 1983a; Ledgard *et al.* 1985b; Boddey *et al.* 1990; Hamilton *et al.* 1992). The estimate may not be correct if the same proportions of soil ^{15}N:^{14}N are not assimilated by the legume and reference plant. This may be due to differences in root distribution or growth rates (Witty 1983a; Chalk 1985; Peoples *et al.* 1989) between the legume and the reference plants.

Natural ^{15}N abundance: The natural abundance of ^{15}N in atmospheric N_2 is 0.3663 atom% ^{15}N (Junk and Svec 1958), while natural abundance of ^{15}N in soil mineral N of most biological systems can be as high as 0.374 atom% ^{15}N. If natural differences in the isotopic composition between soil and atmospheric N are present, the proportion of fixed N can be estimated in the same manner as the ^{15}N-enrichment method, and is called the natural ^{15}N

abundance method. This method has been reviewed by Shearer and Kohl (1986) and Peoples *et al.* (1989). The resulting values are small and therefore the expression delta (δ) ^{15}N (^{15}N ‰) is normally used (Rennie *et al.* 1978). The relative conversion formulae are shown in the section headed"^{15}N Analyses".

This technique is useful because it can be used for an established field trial, because no ^{15}N fertiliser application is necessary and since the natural ^{15}N enrichment is relatively more homogeneous in soil, compared to added ^{15}N-fertiliser as required for the ^{15}N-enrichment method. The major assumption inherent in the ^{15}N-enrichment method, which was that legume and non-fixing reference plant needed have a similar pattern of N assimilation, is of relatively minor importance with the ^{15}N natural abundance method (Bergersen *et al.* 1989; Ledgard 1989). However, some of the limitations of the ^{15}N-enrichment method, are also important for the natural ^{15}N method, when used to estimate legume N_2 fixation in mixed legume/grass pastures. For example, it is assumed that there is no direct transfer of fixed N_2 from the legume to the reference plant (Ledgard *et al.* 1985a) and that there is no N_2 fixation directly associated with the reference plant (Van Berkum and Bohlool 1980).

In the natural ^{15}N abundance method, variation in natural abundance of ^{15}N accumulated in the legume caused by discrimination during N_2 fixation should be measured. Most of the data generally indicate that discrimination against the heavier ^{15}N isotope occurs, i.e. a negative δ ^{15}N value of B is estimated (La Rue and Patterson 1981). Examples of estimates of the isotopic fractionation during N_2 fixation by pasture legumes are given in Table 2.4.

Table 2.4 Isotopic fractionation during N_2 fixation by pasture legumes.

Species	Variety	$B^{\dagger}$	Source
Medicago sativa L.	Mireille	- 0.92	Mariotti *et al* 1980
Trifolium pratense L.	Alpilles	- 0.88	Mariotti *et al* 1980
Trifolium pratense L.		+ 1.88	Kohl and Shearer 1980
Trifolium subterraneum L.	Mt. Barker	+ 2.58	Bergersen and Turner 1983

† = δ ^{15}N (with respect to atmospheric N_2). After Ledgard 1984 (Thesis)

Since the isotopic fractionation during N_2 fixation is probably constant for each legume cultivar and rhizobium species combination, it can be determined by analysis of the nitrogen-fixing plant of interest, growing in N-free medium (Peoples and Herridge 1990).

***^{15}N Analyses*:** Three methods have been used for ^{15}N analyses, viz: Ultraviolet (UV) optical emission spectroscopy, NMR, and mass spectrometry. Only mass spectrometry is sufficiently accurate to measure N isotopes at the level of natural abundance (Shearer and Kohl 1986). Due to the higher demands on accuracy required to measure the small differences in ^{15}N between fixing plant and test plant, the mass spectrometer should be of an isotope-ratio type able to resolve ionised molecules of N_2 into masses 28 ($^{14}N_2$), 29 (^{14}N,^{15}N) and 30 ($^{15}N2$) and to electrically determine the relative concentrations of the ions. It is more accurate if the instrument has a dual inlet system and change-over valve so that the sample and standard gases can be estimated alternately. However, there are a number of new automated nitrogen and carbon analyser mass spectrometers that can achieve high levels of accuracy. Such instruments utilise the Dumas combustion of samples at 1020°C to produce N_2, which is introduced into the connected mass spectrometer as shown in Figure 22.

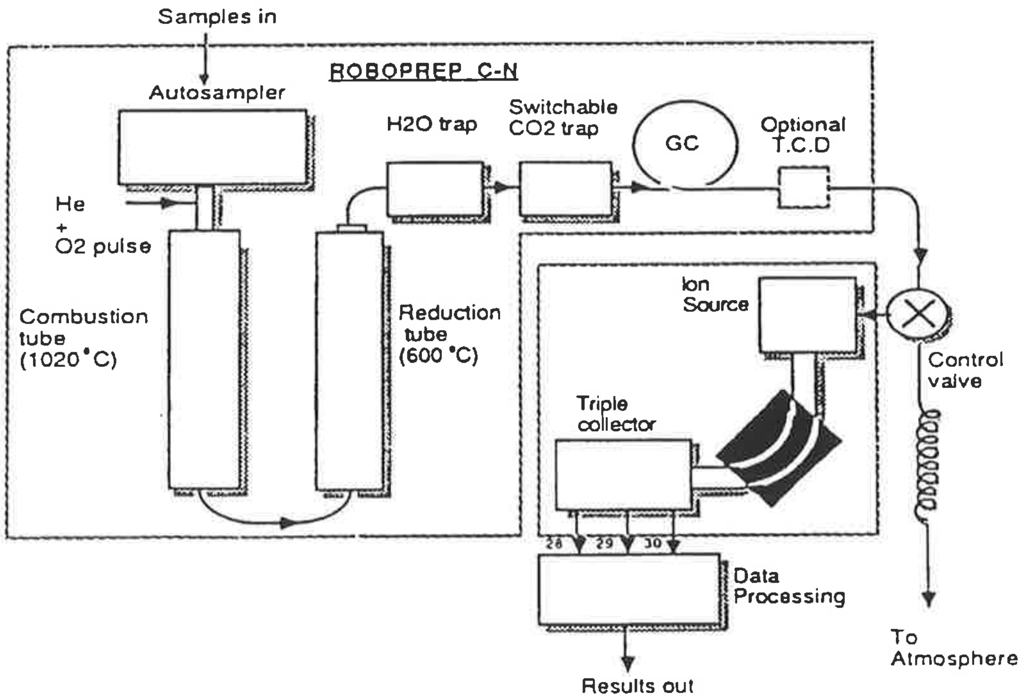


Figure 2.2 Schematic representation of an ANCA-MS, capable of analysing ^{15}N in finely ground plant material. (Source: Hansen 1994).

Errors may arise from poor sample preparation. If the sample is not very finely ground and not homogeneous or if cross-contamination occurs between samples in measurement of δ ^{15}N. Sample sizes are usually small: for most instruments samples are about 0.1- 0.5 mgN (Peoples *et al.* 1989). Due to non-uniform distribution of ^{15}N between plant parts (Bergersen *et al.* 1988), and the distribution of ^{15}N within plant parts also varies with time and experimental conditions it is essential in estimating ^{15}N content to account for these potential sources of error. The equations are as follows:

For ^{15}N enrichment.

When working at natural abundance levels of ^{15}N the data are usually expressed by the more sensitive expression of δ ^{15}N ‰ (Vose and Victoria 1986) where:

$$\text{Atoms\%}^{15}\text{N} = [\frac{^{15}\text{N}/^{14}\text{Nsample}}{^{15}\text{N}/^{14}\text{N standard}} - 1] \text{ x}100$$

The percentage of legume N fixed from atmospheric N_2 (P_{fix}) is calculated from the following formula:

$$P_{fix} = [1 - \frac{\text{Atoms\%}^{15}\text{N excess in fixing plant}}{\text{Atoms\%}^{15}\text{N excess in reference plant}}] \text{ x } 100$$

For Natural ^{15}N abundance

The ^{15}N enrichment relative to that of atmospheric N_2 is given by:

$$\delta\ ^{15}\text{N}(‰) = \frac{\text{Atoms\%}^{15}\text{N sample - Atoms\%}^{15}\text{N air}}{\text{Atoms\%}^{15}\text{N air}} \text{ x } 1000$$

The estimation of P_{fix} is calculated from following equation:

$$P_{fix} = \frac{\delta^{15}\text{N reference plant - }\delta^{15}\text{N fixing plant}}{\delta^{15}\text{N reference plant - }B}$$

where the factor B is the $\delta^{15}N$ value of the same N_2-fixing plants grown in a N-free media (Bergersen *et al.* 1986; Ledgard 1989).

2.3.1.2 Total plant nitrogen

This method relies on measuring the total nitrogen accumulated by the legume during the growing period. The major assumption in measuring N_2 fixation by this method is that all of the legume N has been fixed from the atmosphere, which is usually incorrect under field conditions, because legumes utilise plant-available soil N. Even so, the proportion of legume N fixed from atmospheric N_2 is reported near 90% on soil of low N availability (Ledgard *et al.* 1987). This method may underestimate total fixed nitrogen, when fixed N_2 has been released into the soil by decay and death of legume parts, and/or transferred to the associated grasses in mixed pasture during growth.

Commonly the total N in plants is estimated by the Kjeldahl method (Bergersen 1980b). In this method, organic and mineral N in plant tissues, are reduced to ammonium by heating in concentrated sulphuric acid in the presence of a catalyst. NH_3 is generated from ammonium by alkali, followed by distillation or diffusion. The ammonia is then collected and measured by titration or colorimetrically.

The original Dumas technique, also liberates the N_2 gas from plant material by oxidation in the presence of copper oxide in a sealed glass tube and the amount of N_2 is measured by ultraviolet optical emission spectroscopy (Preston 1991). Estimation by this method is cheaper, but less accurate than other methods.

2.3.1.3 Nitrogen-difference method.

With this method, fixed N is measured by difference in the total amount of N in legume and non-N_2 fixing reference plant grown in the same soil. This technique assumes that the legume and reference plant remove identical amounts of nitrogen from the soil. In field situations, many experiments have indicated that the legume and reference plants do not absorb the same amount of soil N (Hardy and Holsten 1977; and Ledgard *et al.* 1985c). However, the total N methods can by employed by measuring the total N assimilated by plants.

Three approaches to this technique have been used, legume/non-nodulating legume (Vose *et al.* 1982), legume/uninoculated legume (Williams *et al.* 1977), and legume/non-legume (Richards and Soper 1979).

2.3.2 INDIRECT METHODS

2.3.2.1 Acetylene reduction method

The nitrogen-fixing enzyme (nitrogenase) present in the nodule can also reduce acetylene (C_2H_2) to ethylene (C_2H_4) (Koch and Evans 1966; Hardy *et al.* 1968). Briefly, the method involves incubation of the legume in a sealed container with the addition of 10% acetylene for a time period of about 0.5-2 hours. By enclosing the whole plant or plant-soil system in the gas-tight container and by replacing the air in the container by an atmosphere containing acetylene, reduction of N_2 by the nitrogenase is inhibited and instead the enzyme reduces acetylene to ethylene (Hardy and Holsten 1977). After a suitable incubation period, the gas in sampled and analysed for ethylene using gas-liquid chromatography with a flame ionisation detector (Havelka *et al.* 1982).

The acetylene reduction method is cheap, sensitive, rapid and simple (Turner and Gibson 1980). However, there are a number of problems associated with the technique (Ledgard and Steele 1992; Vessey 1994), the important ones being: Acetylene reduction activity by nitrogenase is a short-term measurement, while N_2 fixation normally takes place over long-term periods, and is subject to diurnal, daily and seasonal variations. When calculating N_2 fixation from C_2H_2 reduction, the theoretical ratio of N_2:C_2H_2 differed for different experimental conditions. Hardy *et al.* (1973) suggested conversion ratios of 2.3:1 for soybean, while Ham (1977) reported ratios for soybean ranging from 3.4:1 to 4.4:1. These differences for the same species may reflect different assay conditions Therefore, there is a need to calibrate ethylene production with N_2 fixation. Calibration is necessary for each treatment, for each cultivar, rhizobial strain, growth condition and assay condition (Minchin *et al.* 1994). During the assay, acetylene gas may reduce nitrogenase activity in some legumes (Minchin *et al.* 1983): this means that during the assay a reduced rate of nitrogenase activity is being estimated and leading to an underestimation of N_2 fixation.

It has been suggested (Minchin and Witty 1989) that during an acetylene reduction assay the plant and nodules are disturbed, reducing nitrogenase activity. However, in the review of Vessey (1994) he indicated that the traditional assay with disturbed plant systems can usually be used for comparative purposes. Applying the acetylene reduction method in physiological experiments on intact plants was suggested to have considerable merit (Hunt and Layzell 1993): for example, when estimating nitrogenase activity in response to specific changes in environmental conditions. In addition, the assay has been very useful for qualitative measurements (Hansen 1994).

2.3.2.2 Ureide method

Many tropical and some other legumes with determinate nodules usually transport most of their fixed nitrogen from nodules to the plant in form of ureides whereas non-fixing plants usually transport nitrate taken up by the roots either as nitrate or after conversion to amino acids (Hansen *et al* 1993). The ureides (allantoin +allantoic acid) are found in the nodule tissue as well as in the xylem sap of nodulated legumes (Matsumoto *et al.* 1977). These observations suggested that the relative ureide content of the xylem sap may provide a quantitative measure of symbiotic N_2 fixation (McClure *et al.* 1980; Herridge 1982). This method may be a useful approach to estimate N_2 fixation in those legumes that produce ureides. These are largely the crop legumes, for example soybean (*Glycine max* [L.] Merr) and cowpeas (*Vigna unguiculata* [L.] Walp) (McNeil 1982). In pasture, the legume species such as clover and lucerne produce specific amides (Atkins 1982): therefore, the method may not be suitable for estimating N_2 fixation by these pasture legumes.

Collection of N-solutes such as ureides from plants, is possible by a root-bleeding technique (Norhayati *et al.* 1988), establishment of a mild vacuum to remove tracheal sap (Herridge and Doyle 1988), and harvesting sap via tissue extracts from the stem (Herridge 1982). The N-compounds in xylem sap can be analysed by colourimetric assay (Hansen 1994). There are some limitations with this method, so it is unlikely to be used for quantitative measurement of N_2 fixation by legumes (La Rue and Patterson 1981). It does not provide a time-integrated estimate of N_2 fixation, and as an indirect assay of N_2 fixation, calibration curves, relating ureide production to N fixation must be established for each species (Hansen *et al.* 1993).

2.4. CONCLUSIONS

The specific beneficial role of pasture legumes is mainly related to the biological fixation of nitrogen. This nitrogen fixation has a direct influence in increasing both pasture production and quality in grazed pasture enter prises or in the crop-pasture-livestock farming systems of the creel belt. Proper pasture management can be both informed and responsive to the current requirements.

With pasture management, there is need to understand the functions of nutrients in legumes and nodule formation and activation. The impact of weeds, pests and diseases on pasture productivity and N_2 fixation, needs to be assessed, as does the importance of using integrated weed management, which aims to reduce herbicide use in pasture management.

In addition to the above , the efficient management of N_2 fixation, will also depend upon the ability to estimate N_2 fixation reliably for different pasture conditions. As each method has its own limitations, it is of great importance to recognise such limitations and select the most suitable technique for each specific study.

CHAPTER 3

3.1 EXPERIMENT 1. THE IMPACT OF POST-EMERGENCE APPLICATION OF THE HERBICIDE 2,4-DB ON NODULATION AND NITROGEN FIXATION (AR) OF ANNUAL MEDICS. (Location: Glasshouse at Waite Institute)

3.1.1 INTRODUCTION

In southern Australia, biological nitrogen input from pasture legumes is of fundamental importance to the N economy of crops and pastures in farming systems (Carter 1981), but many of the cereal-cropping areas of the world rely mainly on various sources of industrial nitrogen for maintenance or improvement of soil nitrogen levels. According to Carter (1978) there are 7 million hectares of fallow in the rainfed and irrigated areas in Iran, of which 5 million hectares could be sown to various forage and food legumes leading to increased nitrogen fixation of 300,000 metric tons N for improving soil nitrogen levels for cereal production and giving a potential increase of 22 million metric tons of livestock feed sufficient to support an increased flock of 27.5 million ewe equivalents

However, when legumes are grown in rotation with cereals in Iran, farmers encounter problems will annual grasses and broadleaf weeds. These weeds reduce cereal yields and N input into the soil. Farmers are constantly seeking answers to questions like: how to control the weeds ?, which herbicides to use ?, and when and how to use these herbicides ?.

The control of annual grasses can greatly improve legume herbage and seed production (Carter 1990b) and legume nitrogen fixation (Butler 1987). The grass control also improves the health and production of prime lambs (Little *et al.* 1992). Furthermore, the control of annual grasses reduces the incidence of cereal root diseases in the following cereal crop (Inwood *et al.* 1992).

Reports from several workers indicate that herbicides reduce nodulation and N_2 fixation in legumes (Fletcher *et al.* 1956; Torstensson 1975; Eberbach and Douglas 1983; Cardina *et al.* 1986; Eberbach and Douglas 1989). There have been several verbal reports by agronomists of the South Australian Research and Development Institute (SARDI), fertiliser companies and farmers that when the herbicide 2,4-DB is used to control broadleaved weeds in medic pasture in South Australia, nodulation of young medic plants is reduced. As a result 2,4-DB is not used widely in other parts of Australia and has been replaced commercially by cheaper herbicides (Schrodter *et al.* 1984; Cudny *et al.* 1993). However, 2,4-DB is still used extensively by farmers in South Australia, particularly for annual medic seed production.

The variation in tolerance of annual pasture legumes to herbicides has been evaluated by a number of workers. For example, Mulholland *et al* . (1989) examined the effects of the herbicide 2,4-DB on growth of.annual medics and found significant differences in response. Variable tolerance to 2,4-DB in a range of subterranean clover cultivars has also been reported by Evans *et al.* (1989) and Young *et al.* (1992), while Conlan *et al.* (1990) reported a significant difference in the response of subterranean clover cultivars to simazine.

Attempts to improve the nitrogen-fixing capacity of legumes through species selection is complex because there are many components to consider, including plant persistence, growth rate and ability to nodulate and fix nitrogen. According to Eberbach and Douglas (1989) the reduction in nodulation of the legume may be the result of physiological damage to the root system or to the *Rhizobium* before or during infection. Thereafter, a decline in nitrogenase activity may result from effects on root nodules or from a reduction in supply of photosynthate to the nodules. A direct linear relationship between yield of annual medics and amount of fixed N has been shown by Butler (1987) and confirms that the capacity to fix N_2 varies among the species and cultivars through effects on dry matter production.

According to Carter (1987) the persistence of annual legumes can be improved by selecting better species and genotypes, and by improving pasture management. One of the characters that may be sought, to improve persistency and capacity to fix N_2, is herbicide tolerance. Annual medics have been shown to be susceptible to many types of herbicides. Since variation in herbicide-tolerance amongst cultivars of one species, or closely-related species, is common (Mulholland *et al.* 1989), the selection of herbicide-resistant species of annual legumes will be useful in enhancing the biological N_2 fixation in pastures.

The research detailed in this chapter aims to investigate the effects of the herbicide 2,4-DB on the growth, nodulation and N_2 fixation of 10 species and cultivars of annual medics.

3.1.2 MATERIALS AND METHODS

3.1.2.1 Plant material

Ten cultivars of four annual medic species, namely: *Medicago truncatula* cvv. Caliph, Jemalong, Mogul, Parabinga and Paraggio, *M. littoralis* cvv. Harbinger and Harbinger(AR), *M. scutellata* cvv. Kelson and Sava and *M. rugosa* cv. Paraponto were chosen for this experiment. These species and cultivars represent commercial Australian medics adapted to southern Australia.

3.1.2.2 Plant culture

Seed was sown on 24 April 1992 in washed, coarse river sand in square (15 × 15 cm) black plastic pots of 2 L capacity with holes near the base. Seeds were inoculated at time of sowing with a peat inoculant (*Rhizobium meliloti* strain AM WSM 540), mixed with deionised water to form a slurry. A second inoculation was made 1 week after sowing through the irrigation water, to ensure effective nodulation. A total of 50 seeds per pot were sown directly into the pots using a template with 25 equally spaced holes and 2 seeds per hole. Seedlings were thinned to achieve a density of 25 plants per pot 15 days after sowing and soon developed into a sward. Pots were irrigated with rain water until emergence (after 3 days) and thereafter watered to field capacity (11%) with nutrient solution each morning followed by the addition of the plant-required amount of rain water in the afternoon. Nutrient solution contained no nitrogen but included (mg/L) $CaSO_4$ $2H_2O$ (430), KH_2PO_4 (33.75), K_2SO_4 (217.8), $MgSO_4$ $7H_2O$ (245), $FeSO_4$ $7H_2O$ (39.8), EDTA (47.64), $MnCl_2$ $4H_20$ (1.81), $ZnSO_4$ $7H_2O$ (0.22), $CuSO_4$ $5H_2O$ (0.08), Na_2Mo O_4 $2H_2O$ (0.12), H_3 BO_3 (2.86) prepared from stock solutions with deionised water. KOH was used to adjust the pH of the solution to 7.0.

Plants were grown in a glasshouse under natural illumination and temperature varying between a mean monthly minimum of 14-15°C and a mean monthly maximum of 22-30°C.

The positions of pots were regularly re-randomised on the glasshouse bench. At 21 days after emergence, wire-mesh sleeves (12 cm high) were fitted to the pots to confine developing leaf canopies to a constant area of the pot surface.

3.1.2.3 Design and treatments

The experiment was a factorial design comprising 10 species and cultivars of medics randomised in two blocks. Treatments comprised two times of application of herbicide 2,4-DB (at the first or fifth trifoliate leaf stages i.e. 1TLS or 5TLS) at three rates, viz: control sprayed with deionised water, half the recommended rate (1.4 L/ha) and the recommended rate (2.8 L/ha). Thus there were a total of 120 pots. A laboratory pot sprayer moving at a constant speed 40 cm above the pots with a pressure of 250 k Pa and an output of 117 L/ha was used to spray the herbicide. Plants were not irrigated for 24 hours after spraying.

3.1.3 DATA COLLECTION

The sward was sampled for total dry matter (shoot and root), nodule number and nitrogenase activity 24 days after both herbicide application times.

3.1.3.1 Estimation of nitrogenase activity

Nitrogenase activity was estimated by the acetylene reduction assay using a closed system. The assay is based on the ability of the nitrogenase enzyme to reduce acetylene (C_2H_2) to ethylene (C_2H_4). According to Shearer and Kohl (1986), this method is suited to the detection and comparison of nitrogen fixation after different treatments. However the closed system of assay has been criticised by several workers as underestimating the actual rate of ethylene production due to a disturbance of environmental conditions for the N_2 fixation organism during assay (Hardy *et al.* 1968, 1973; Bergersen 1970; Postgate 1971; Watanabe and Cholitkul 1979). To overcome this problem a modified system was designed in collaboration with Dr. A. Gibson of the Division of Plant Industry, CSIRO, Canberra. Plants in each pot were covered and sealed with another pot from which the bottom had been replaced with a sheet of transparent plastic 2 mm thick, and fitted with a rubber septum (Plate 3.1.1). An injection of 200 ml acetylene through the top (100 ml) and base (100 ml) of each pot was made using 100 ml syringes fitted with a needle to give a partial pressure of 0.1 mm C_2H_2. Excess gas was allowed to escape through an extra needle to prevent pressure build up in the pot. The rate of acetylene reduction activity (AR) was calculated from the C_2H_4 concentration in 500 µl gas samples taken at 15 and 45 minutes after C_2H_2 was added using 1 ml syringes each fitted with a needle.

Plate 3.1.1 Modification of assay system by covering the pot and plants with another transparent pot.

The samples were injected into a Varian Aerograph model 940 gas chromatograph equipped with a flame ionisation detector. Ethylene concentration was estimated from peak height displayed on a flat bed omniscribe recorder (Houston Instruments). Standards of C_2H_4 (200 µl) (7% acetylene in air at 20°C) were made up in a standard pot (same as the sample pots without plants). The differences in peak heights between the average of three sample injections taken at 15 minutes and 45 minutes were converted to µ mol C_2H_4 / m^2 / h by reference to peak height injections from standards of known quantity of C_2H_4.

The rate of C_2H_4 production was calculated from the following equation:

$$\mu\text{mol } C_2H_4 = \frac{Q \times D \times P \times 2}{V \times G \times S} \times 44.4$$

Where Q = quantity of C_2H_4 in air within a standard pot at 20°C; V = volume of standard pot (ml); D = difference in peak heights between 3 sample injections taken at 15 and 45 minutes; P = volume of gas in assay pot; G = volume of 1 mole of gas at assay temperature; S = peak height of standard and 44.4 was the factor converting pot results to a m^2 basis.

3.1.4.2 Dry matter production and nodule score

After the estimation of nitrogenase activity from a total of 30 pots (one of the replicates from one of the herbicide spraying times), one for each cultivar and treatment, were carefully washed with water to separate the plants from the sand. Nodule numbers on the top and bottom halves of the roots were counted. Very small nodules (not detachable with ease) were not counted. Generally nodule size was small in all treatments. Dry weights were determined after drying plant fractions in a forced draught oven at 85°C for 24 hours. Plant densities were determined by counting the plants in each pot.

3.1.3.3 Statistical analyses

The data were analysed using standard analysis of variance with repeated measures, and where the t-test indicated a significant difference ($P<0.05$) between means, t-test least significant differences (LSD) were calculated at the 5% level (Steel and Torrie 1960). Results from each time of spraying were analysed separately, because at each time of spraying the plants were at different stages of growth (1TLS and 5TLS), so that cultivar differences for each rate of herbicide application could be seen for each time of herbicide application. The appropriate data were also analysed using regression analysis.

3.1.4 RESULTS

3.1.4.1 Visual assessments

Most treated plants were dark green and slightly scorched compared to control plants, particularly at the full rate of herbicide application. Wilting or death of some petioles and leaflets of plants occurred in Harbinger, Kelson and Paraponto when sprayed at the 1st trifoliate leaf stage (1TLS). Similar results were obtained in Mogul and Kelson after the late application of herbicide at the 5th trifoliate leaf stage (5TLS). Generally, foliar damage two weeks after spraying was greater after the early application than after the later application of herbicide.

3.1.4.2 Plant densities

Herbicide application caused the death of whole plants in some of the cultivars at both times of spraying. The reduction in plant density was significant ($P<0.05$) only in Kelson at both times and rates of herbicide application (Table 3.1.1). In this case the reduction in plant density was severe.

Table 3.1.1 The effects of the herbicide 2,4-DB sprayed at three rates of application [zero, half (1.4 L/ha) and the recommended rate (2.8 L/ha)] at the 1st or 5th trifoliate leaf stage on the plant population density of ten annual medics sampled 4 weeks after each spraying.

Species and cultivars	**Treatments**					
	Early sprayed (1TLS)			Late sprayed (5TLS)		
	Control	Half rate	Full rate	Control	Half rate	Full rate
M. truncatula						
Caliph	25	23	25	25	25	22
Jemalong	25	25	23	25	24	23
Mogul	25	24	24	25	22	18
Parabinga	25	24	25	25	23	25
Paraggio	25	25	24	25	25	23
M. littoralis						
Harbinger	25	25	17	25	25	23
Harbinger(AR)	25	23	24	25	25	25
M. scutellata						
Kelson	24	15	3	25	6	2
Sava	25	23	21	25	24	22
M. rugosa						
Paraponto	25	22	21	25	24	23
LSD (5%)		9			6	

3.1.4.3 Shoot dry weight

When the medic plants were harvested, shoot and root dry matter yields were determined and statistically analysed. There were significant interactions between cultivar and herbicide for shoot dry weight irrespective of the rate of herbicide used. A highly significant response of shoot dry matter for the different rates of application and different cultivars was also observed (Table 3.1.2).

Table 3.1.2 Summary of ANOVA of the effects of the cultivars, rate of the herbicide application and time of spraying on plant yield (kg DM/m^2) of the medic cultivars .

	Dry matter yield		
Source of variation	Shoots	Roots	Total plant
	(Significance levels)†		
Rate of herbicide	***	***	***
Cultivars	***	NS	***
Time of spraying	NS	NS	NS
Rate of herbicide × Cultivars	**	***	***
Rate of herbicide × Time of spraying	NS	***	**
Cultivars × Time of spraying	NS	NS	NS
Rate of herbicide × Cultivars × Time of spraying	NS	*	NS

† NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Over all cultivars, shoot dry matter yields were significantly (P<0.05) reduced by the herbicide application through a retardation in growth and reduction in plant population density of some cultivars at both the early and later applications compared with controls. The effects of the herbicide differed significantly (P<0.05) between the full and half rates of application at the first trifoliate leaf stage only (Table 3.1.3).

Table 3.1.3 Overall effects of the herbicide 2,4-DB sprayed at the 1st or 5th trifoliate leaf stage on shoot dry matter yield of annual medics and sampled 4 weeks after each spraying.

	Shoot DM (g/m^2)	
Herbicide rate (L/ha)	1st trifoliate leaf stage	5th trifoliate leaf stage
0	109.1	160.0
1.4	77.6	110.9
2.8	62.9	106.2
LSD (5%)	13.6	16.3

Shoot dry matter production differed between species and cultivars. In the untreated controls at the first harvest shoot dry matter was highest in Paraggio (135.6g/m^2) and lowest in Harbinger (44.4g/m^2) (Plate 3.1.2).

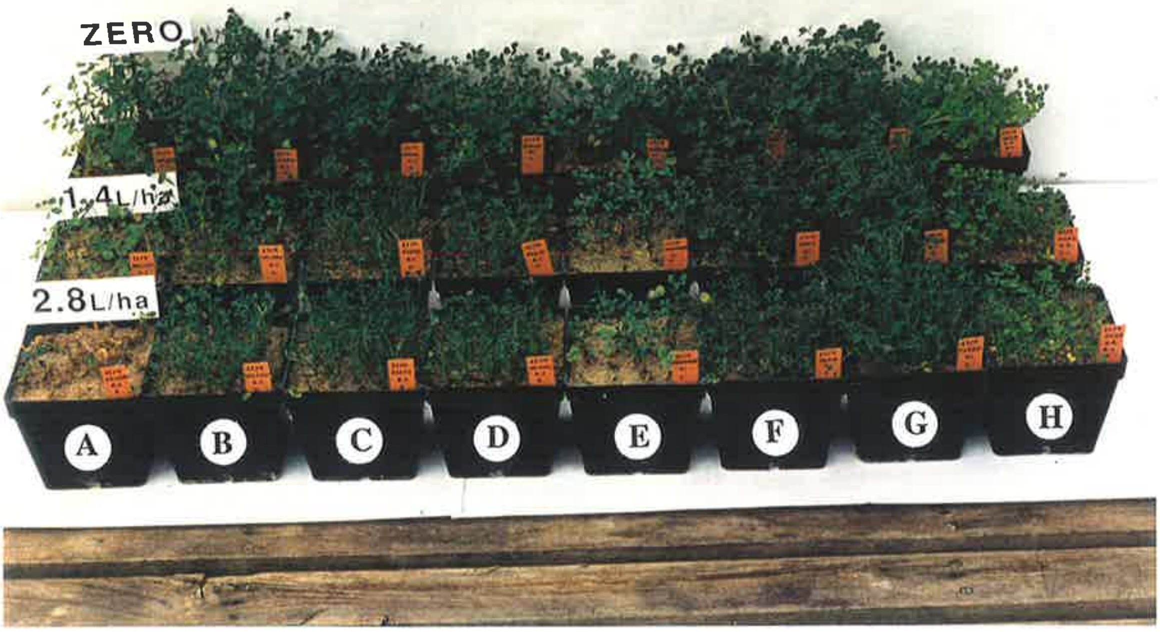


Plate 3.1.2 Effects of the herbicide 2,4-DB applied at the 1TLS on medic growth 24 days after spraying. A = Kelson, B = Caliph, C = Paraggio, D = Mogul, E = Harbinger F = Jemalong, G = Parabinga, H = Harbinger (AR).

At the second harvest Sava was superior in dry matter production, but Harbinger remained the lowest (Plate 3.1.3).

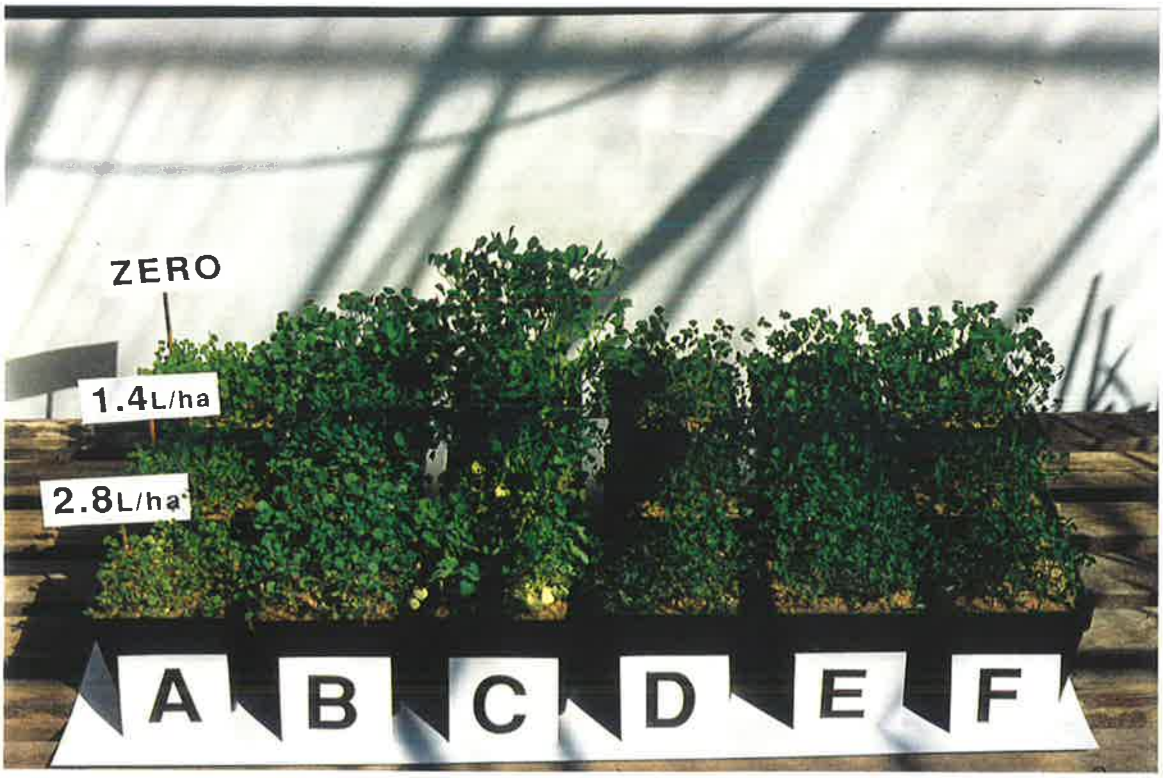
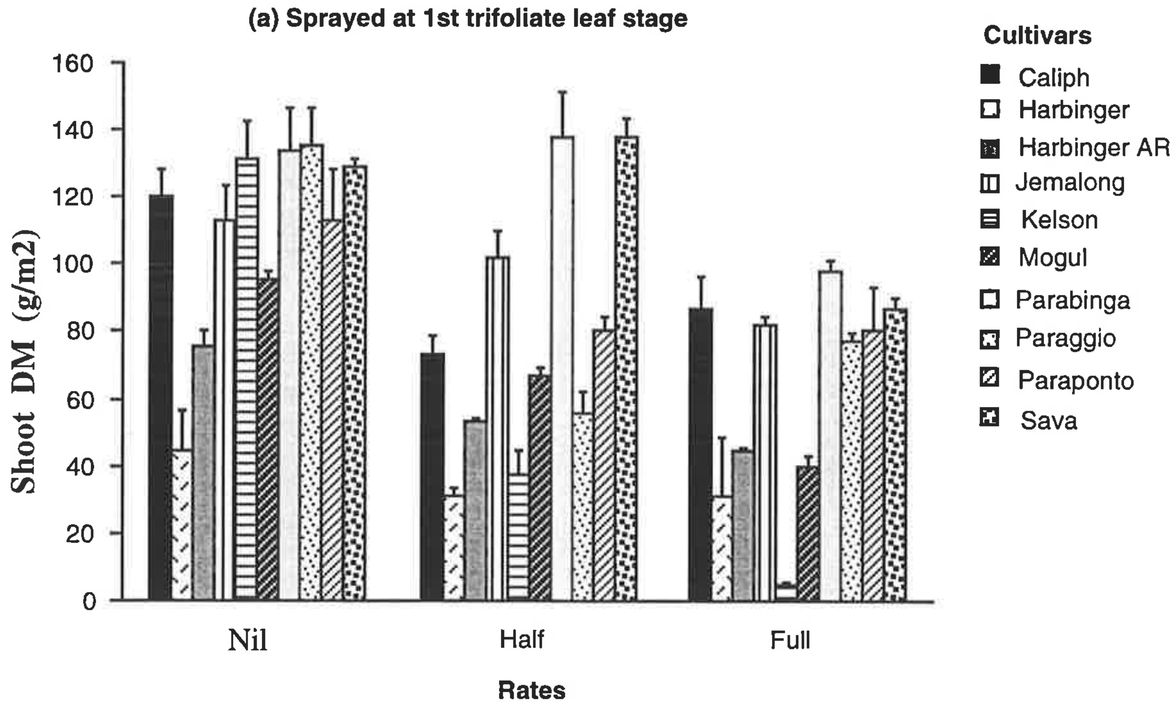


Plate 3.1.3 Effects of the herbicide 2,4-DB applied at the 5TLS on medic growth 24 days after spraying. A = Harbinger, B = Caliph, C = Sava, D = Jemalong, E = Paraggio, F = Mogul.

The plants differed in their response to the herbicide sprayed at different rates and times. Harbinger was more tolerant of 2,4-DB when sprayed at 1TLS, while Caliph was the only tolerant plant when the herbicide was sprayed at the 5TLS. Shoot yield in Harbinger (AR) Kelson, Mogul, Paraggio and Paraponto was reduced by both rates of the herbicide at both times of the herbicide application. Application of the herbicide significantly ($P<0.05$) reduced shoot dry matter yield at recommended rates in most cultivars, while the reduction was significant in Jemalong only at half rate of herbicide application at the 5TLS. Harbinger recorded a significant ($P<0.05$) reduction in shoot yield at both rates of herbicide only at the 5TLS, but not at the 1TLS (Figure 3.1.1). These differential effects suggest the possibility of selection of the annual legumes for tolerance to the herbicide 2,4-DB.



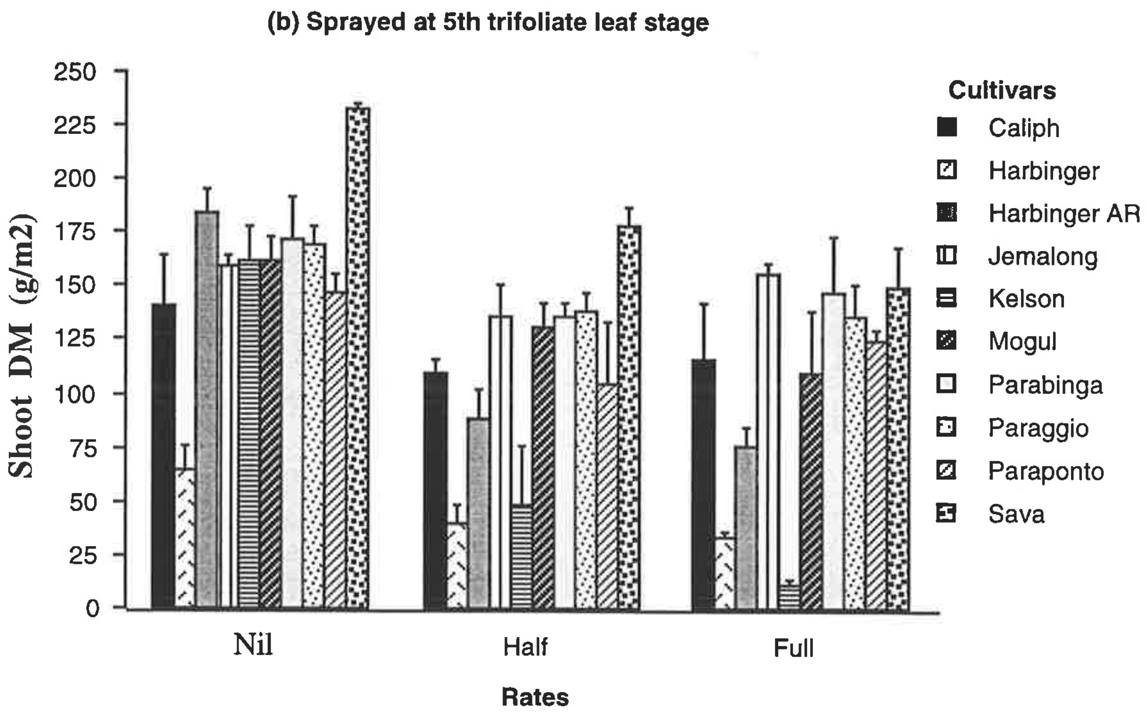


Figure 3.1.1 Shoot dry matter production of 10 annual medics sprayed with 2,4-DB at three rates of application [Nil, half (1.4 L/ha) and the recommended rate (2.8 L/ha)] at 1TLS or 5TLS.
(Plant sampled 4 weeks after spraying. Bars indicate the standard errors of the means)

3.1.4.4 Root dry weight

The analyses of variance of root DM values showed a highly significant effect of herbicide rate of application and the interaction between rate of herbicide and cultivars. There was also a highly significant effect on the time of the herbicide application on the medic root weights. The interaction Rate of herbicide × Cultivars × Time of spraying was significant for plant roots, although the main effects of cultivars and time of spraying on root weight was not significant (Table 3.1.2).

Over all cultivars, root dry weight was significantly ($P<0.05$) reduced at both rates of the herbicide application at both times of spraying. Significant differences in root dry weight between the full and half the recommended rate of the herbicide were found only when the herbicide was sprayed at the 1st trifoliate leaf stage (Table 3.1.4).

Table 3.1.4 Overall effects on medic root yield of the herbicide 2,4-DB sprayed at three rates of application at the 1st or 5th trifoliate leaf stage.

	Root DM (g/m^2)	
Herbicide rate (L/ha)	1st trifoliate leaf stage	5th trifoliate leaf stage
0	86.4	141.1
1.4	72.0	92.7
2.8	61.6	98.9
LSD (5%)	9.7	12.1

Root dry weight varied between plant species and cultivars. In untreated plants at the 1st trifoliate leaf stage, Kelson had the highest root dry weight (120 g/m^2) but this dry weight was significantly reduced when the herbicide was sprayed at either rate of application. The full rate of herbicide gave the lowest dry matter yield of 4.4 g/m^2. The reduction in root dry matter in Kelson by the herbicide application appeared also at the 5TLS, because of the death of a number of plants due to the herbicide. Root weight in Harbinger (AR), Kelson and Mogul was significantly reduced by herbicide spraying at both rates and times. Root dry matter in Paraponto was significantly reduced at the both rates of herbicide at the 1st trifoliate leaf stage only but was not affected at the 5TLS. This was opposite to Harbinger, Parabinga and Paraggio where roots weight was reduced significantly by both rate of herbicide application at the 5th trifoliate leaf stage and did not change at the 1TLS. In some cultivars such as Caliph and Jemalong, root dry weight did not show marked differences after herbicide application at either rates and times of spraying (Figure 3.1.2).

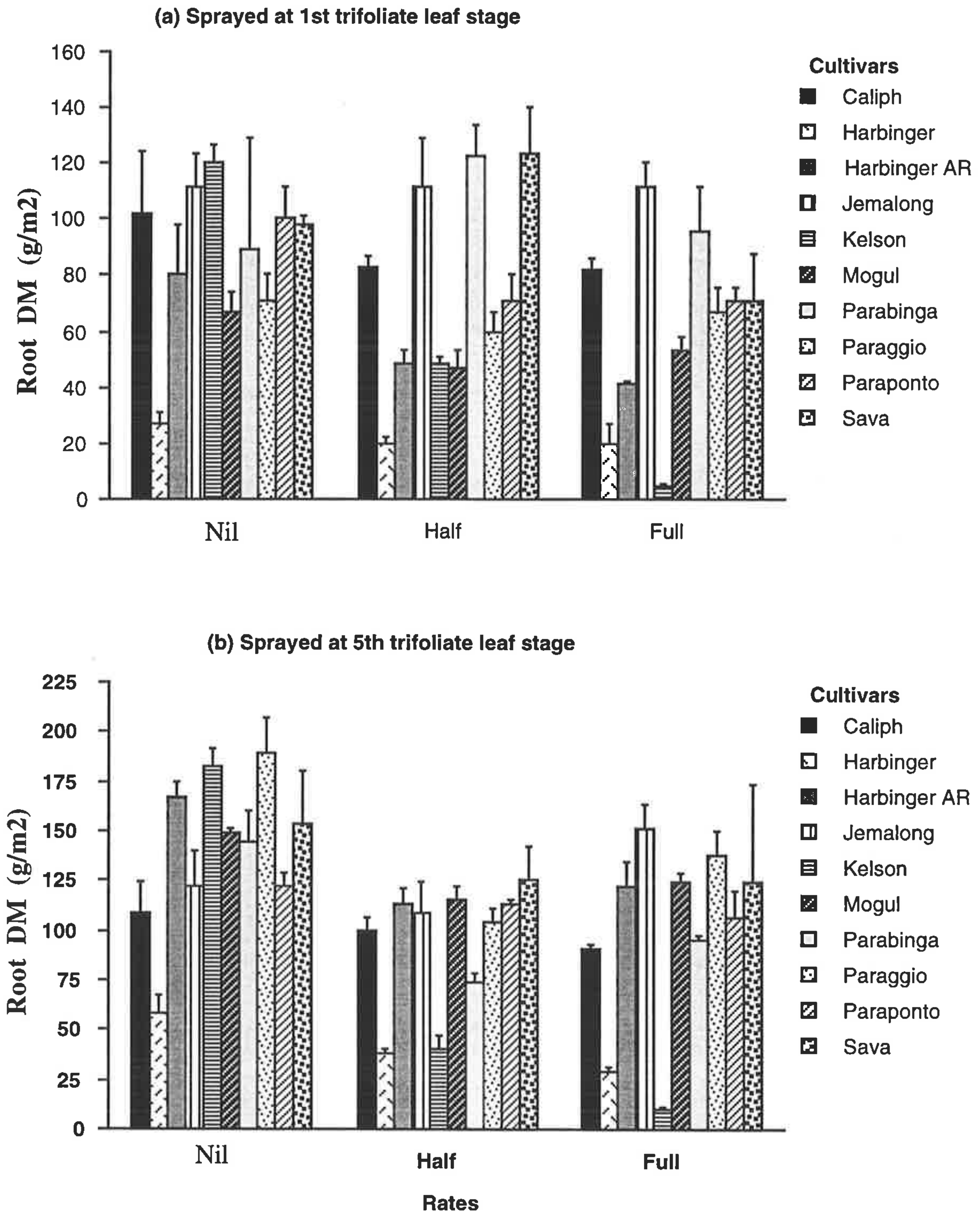


Figure 3.1.2 **Root dry matter production of ten annual medics four weeks after spraying with 2,4-DB at three rates at the 1st and 5th trifoliate leaf stages.** (Bars show standard errors of the means).

3.1.4.5 Nodule number

Analyses of variance were carried out on the data for nodule numbers of all cultivars (Table 3.1.5). There was a significant interaction between cultivars and rates of herbicide for upper nodule number in the upper root zone.. Herbicide application was found highly significant in reducing nodule number. In the case of nodule numbers in the upper root zone there was a significant Cultivars × Time of spraying interaction and there was a significant difference in nodule number between the cultivars.

Table 3.1.5 Summary of ANOVA of the effects of cultivars, rate of the herbicide application and time of spraying 2,4-DB on nodule number per plant and AR activity (μmol $C_2H_4/m^2/h$) of the medic cultivars.

Source of variation	Upper nodules (#/plant)	Lower nodules (#/plant)	Total nodules (#/plant)	AR activity (μmol $C_2H_4/m^2/h$)
		(Significance levels)†		
Rate of herbicide	***	NS	***	***
Cultivars	NS	*	NS	*
Time of spraying	NS	NS	NS	NS
Rate of herbicide × Cultivars	*	NS	*	*
Rate of herbicide × Time of spraying	NS	NS	*	NS
Cultivars × Time of spraying	NS	*	*	NS
Rate of herbicide × Cultivars × Time of spraying	NS	NS	*	NS

† NS = Not significant, * = P<0.05, *** = P<0.001

When all cultivars are considered the total nodule number was significantly reduced (P<0.05) by the herbicide at both rates of application and at both times of spraying (Table 3.1.6).

Table 3.1.6 Overall effects of the herbicide 2,4-DB sprayed at three rates at the first or fifth trifoliate leaf stage on total nodule number (per plant) of annual medics.

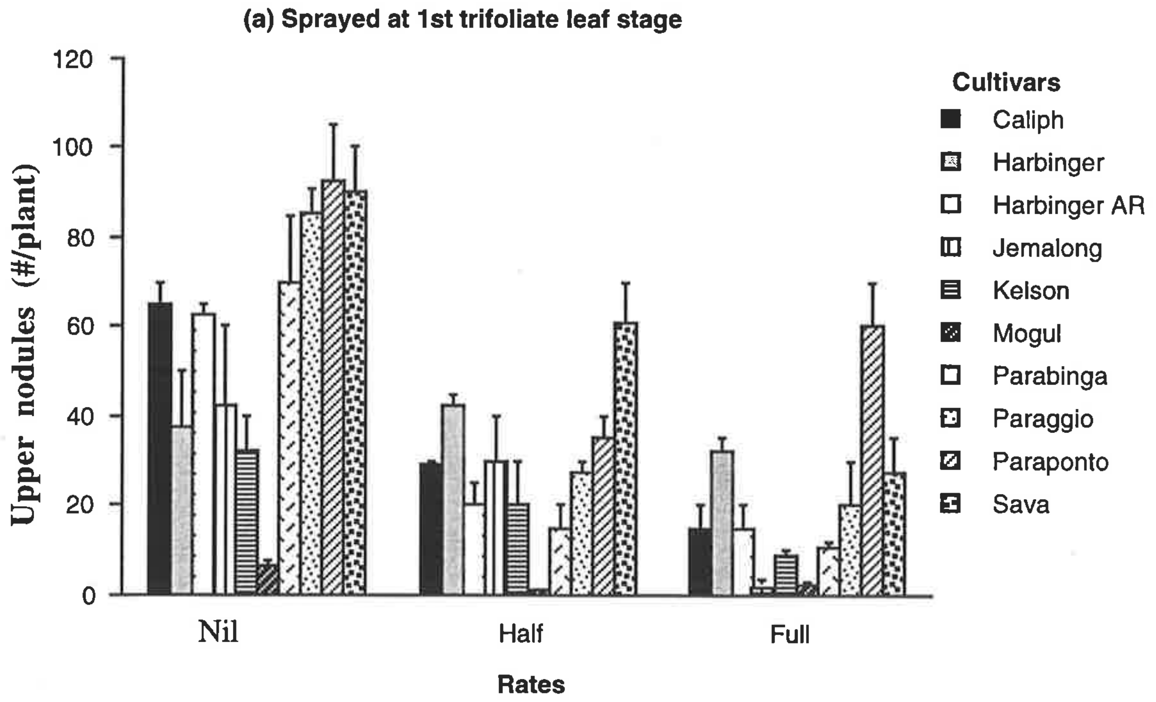
	Nodule numbers per plant	
Herbicide rate (L/ha)	1st trifoliate leaf stage	5th trifoliate leaf stage
0	81	126
1.4	61	77
2.8	47	69
LSD (5%)	16	16

For all cultivars nodule numbers on the upper half of the root were significantly reduced ($P<0.05$) by both rates of the herbicide and times of spraying. Nodule numbers on the lower half of the roots were not significantly affected by the herbicide at both rates and times of application (Table 3.1.7).

Table 3.1.7 Overall effects of the herbicide 2,4-DB sprayed at three rates at the first or fifth trifoliate leaf stages on nodule numbers (per plant) on the upper and lower half of the roots of medics.

	Total nodule number			
Herbicide rates	1st trifoliate leaf stage		5th trifoliate leaf stage	
(L/ha)	Upper	Lower	Upper	Lower
0	58.2	22.9	81.7	44.6
1.4	27.7	32.9	36.7	40.3
2.8	19.4	27.3	25.3	43.8
LSD (5%)	12.4	NS	10.7	NS

Although nodule numbers on the upper portion of the roots were lower than the control at both rates and times of herbicide application, a significant reduction in nodule numbers by the herbicide were found only for Harbinger (AR), Mogul, Parabinga, Paraggio, Paraponto and Sava at both rates and times of application. In Kelson the full rate of herbicide significantly reduced upper nodule numbers at both times of application. In Caliph the nodule number was reduced markedly by the herbicide at 1TLS, while in Harbinger nodule numbers were only significantly reduced by both rates of the herbicide at the 5TLS (Figure 3.1.3).



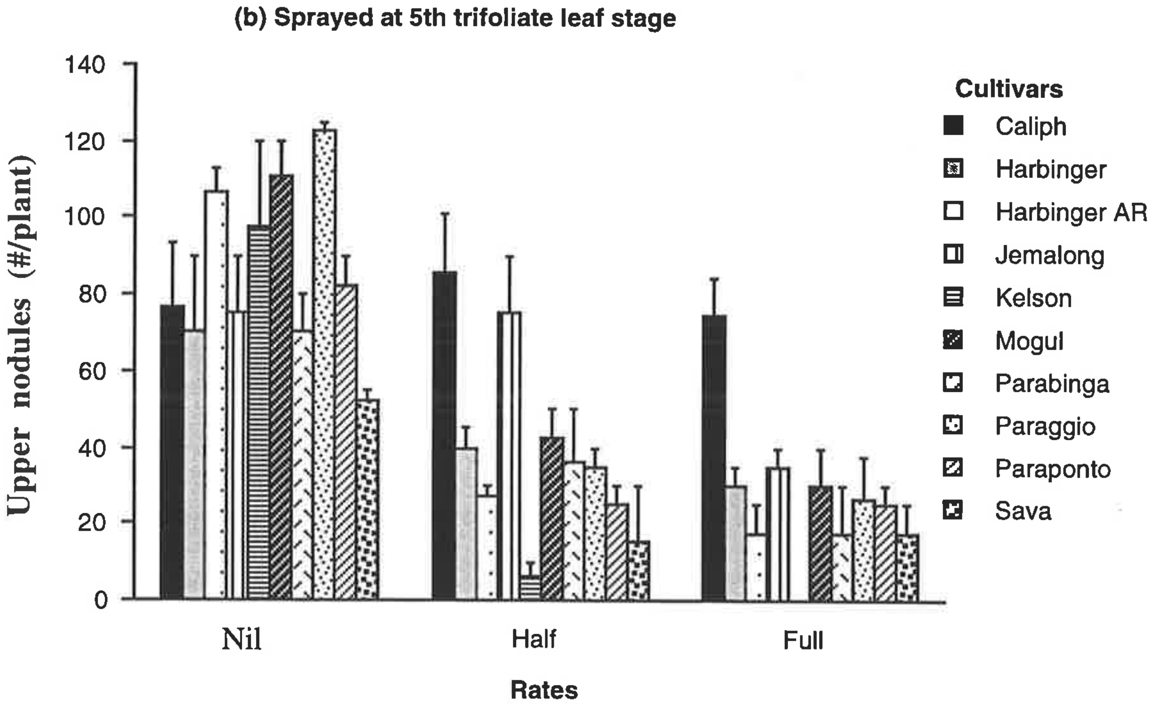
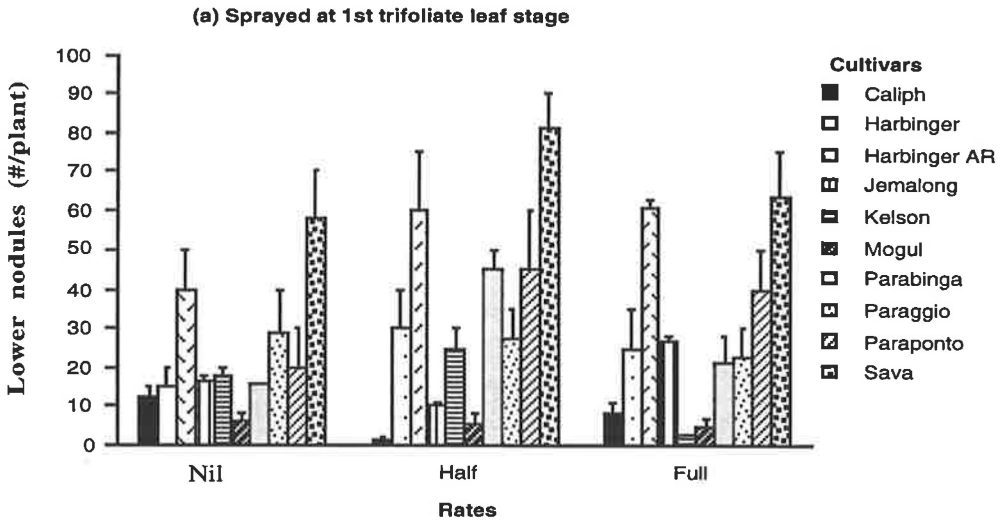


Figure 3.1.3 Upper nodule number (per plant) of 10 annual medics sprayed with 2,4-DB at three rates at the first or fifth trifoliate leaf stage.
(Plants were sampled 4 weeks after spraying, Bars indicate the standard errors of the means).

Nodule numbers on the lower half of the roots of cultivars generally were not significantly affected at either rate of herbicide at both the 1st and 5th trifoliate leaf stages. The exceptions were Kelson at full rate and in Caliph and Jemalong at half rate of herbicide application observed at the 1st trifoliate leaf stage. In Harbinger (AR) and Kelson the lower nodule number was significantly ($P<0.05$) reduced by both rates of the herbicide at the 5th trifoliate leaf stage only. Parabinga and Mogul showed a significant increase in nodule number following herbicide application at the 1st and 5th trifoliate leaf stages respectively (Figure 3.1.4). The lack of effect on nodule numbers on the lower part of the roots, presumably indicates the recovery of the plants from the herbicide application and growth of nodulated roots



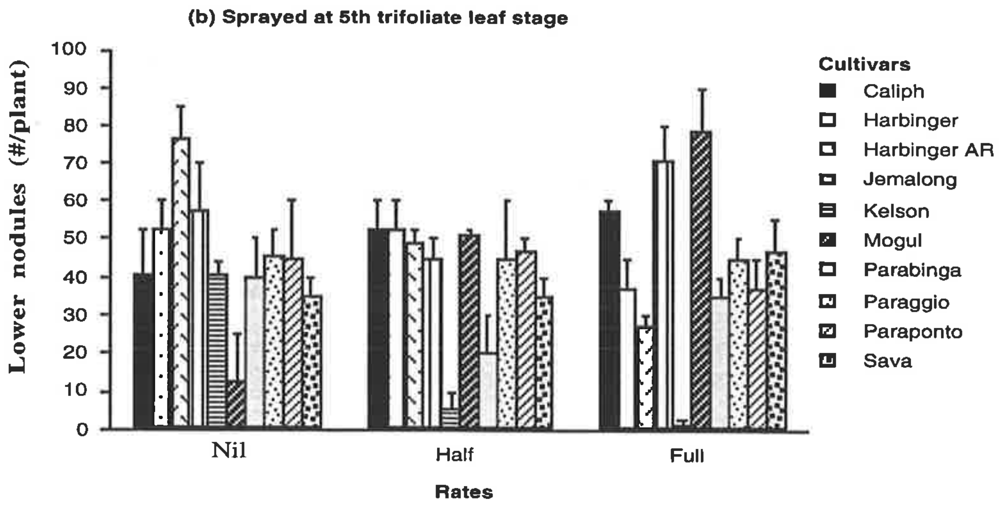


Figure 3.1.4 **Lower nodule number (per plant) of 10 annual medics sprayed with 2,4-DB at three rates at the 1st trifoliate leaf stage or the 5th trifoliate leaf stage.** (Plants were sampled 4 weeks after spraying. Bars indicate the standard errors of the means).

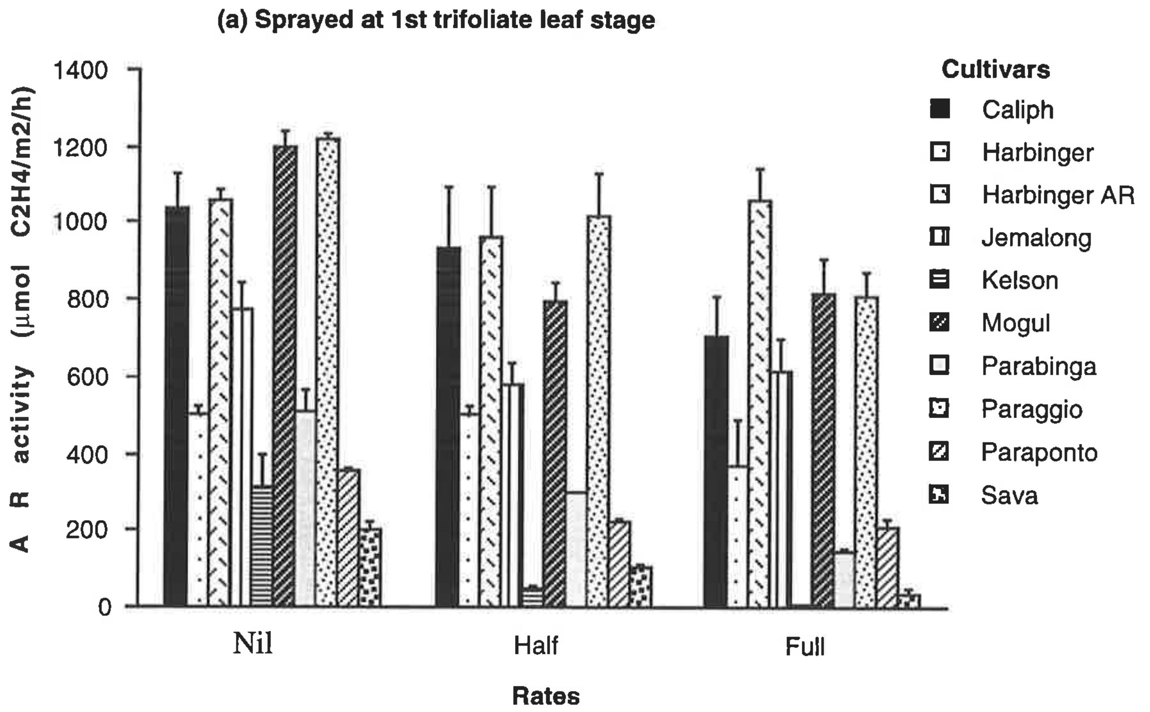
3.1.4.6 Acetylene reduction (AR)

The rate of the herbicide application had a larger influence on AR activity of the medic sward than did time of spraying of the herbicide (Table 3.1.8). A single main effect of cultivars and Rate of herbicide × Cultivar interaction was also observed for AR activity. When considering all cultivars at both times of spraying, the half and full rates of the herbicide significantly ($P<0.05$) reduced AR activity compared with controls. While a significant difference ($P<0.05$) was found at the 5TLS between the half rate and recommended rate of herbicide, the AR activity at the two rates of herbicide application did not differ significantly at the 1TLS. (Table 3.1.8)

Table 3.1.8 Overall effect of the herbicide 2,4-DB sprayed at three rates of application at the 1st or 5th trifoliate leaf stage on nitrogenase activity (μmol $C_2H_4/m^2/h$) of the medics 24 days after spraying.

	Nitrogenase activity (μmol $C_2H_4/m^2/h$)	
Herbicide rate (L/ha)	1st trifoliate leaf stage	5th trifoliate leaf stage
0.0	715	763
1.4	541	584
2.8	487	508
LSD (5%)	75	72

AR activity differed significantly ($P<0.05$) between species and cultivars. In untreated controls, Paraggio had the highest activity of 1220 μ mol $C_2H_4/m^2/h$ at the first harvest four weeks after spraying at the 1TLS and Mogul of 1303 μ mol $C_2H_4/m^2/h$ at the second harvest, four weeks after spraying at the 5TLS. The AR activity was significantly ($P<0.05$) reduced in most cultivars namely Jemalong, Kelson, Mogul, Paraponto and Sava by both rates of herbicide application at both times of spraying. The reduction was significant in Caliph only at the full rate of herbicide at both times of the application. Harbinger showed a reduction in AR activity at the full rate and Parabinga by both rates of herbicide at the 5TLS, but not at the 1TLS. Kelson recorded the lowest C_2H_2 reduction at both times of herbicide application. In this experiment AR activity in Harbinger (AR) did not show any significant reduction following herbicide spraying at both times of herbicide application (Figure 3.1.5).



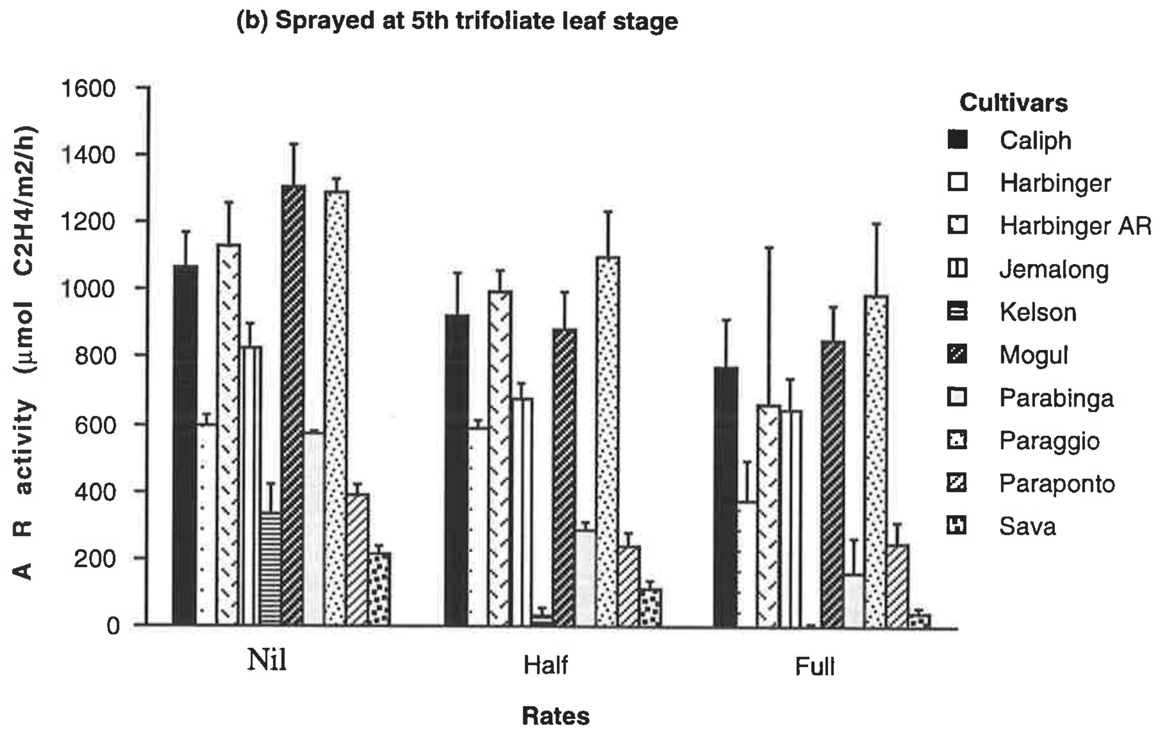


Figure 3.1.5 **Nitrogenase activity (µmol $C_2H_4/m^2/h$) of ten cultivars of annual medics sprayed with 2,4-DB at three rates of application at the 1st or 5th trifoliate leaf stages.**
(Plants were sampled 4 weeks after spraying. Bars indicate the standard errors of the means).

Simple linear regression analyses were carried out on the data from all cultivars, in order to detect which of the measured plant properties correlated with AR activity, and correlated with each other at each time of herbicide application. The relationship between AR activity and total nodule number and also between total plant dry weight and total nodule number was not significant over all cultivars at the 1TLS: however, the relationships were significant at the 5TLS (Figures. 3.1.6 and 3.1.7).

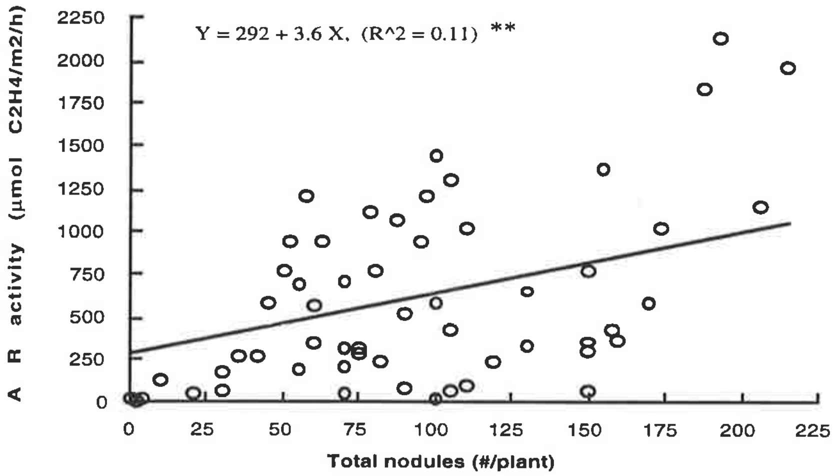


Figure 3.1.6 **The correlation between acetylene reduction activity and nodule number in medic sprayed with 2,4-DB herbicide at the 5th trifoliate leaf stage.**

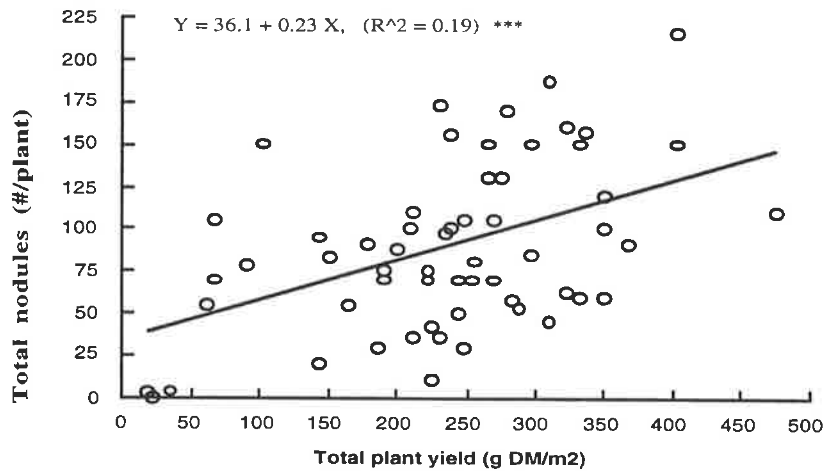


Figure 3.1.7 **The correlation between nodule number and plant yield (shoots+roots) in medic sprayed with 2,4-DB herbicide at the 5th trifoliate leaf stage.**

3.2 EXPERIMENT 2. EFFECTS OF A RANGE OF PRE-EMERGENCE HERBICIDES ON YIELD AND NODULATION OF ANNUAL MEDICS AND SUBTERRANEAN CLOVER.

(Location: Field at Turretfield Research Centre, Rosedale, S.Australia)

3.2.1 INTRODUCTION

In the previous experiment, the post-emergence herbicide 2,4-DB was sprayed on medic seedlings at the first trifoliate leaf stage (during nodule formation) and fifth trifoliate leaf stage (when most nodules were formed), under glasshouse conditions. Results showed that plant weight, nodule number and efficiency in the medic species were adversely affected (but to differing degrees) by different concentrations of the herbicide. Since farmers have already started using other herbicides to overcome the adverse effects of 2,4-DB, a field experiment was conducted to examine the effects of a number of pre-emergence herbicides on dry matter production and nodulation of some pasture legumes. In the review by Martensson and Nilsson (1989), they indicated that herbicides have different effects on nodule formation and activity of legumes. While some herbicides do not exert any influence on nodulation, others exert short - or long-term inhibitory effects on the symbiotic relationship. Other investigators (Cardina *et al.* 1986; Moorman 1986; DeFelipe *et al.* 1987; Fabara De Peretti *et al.* 1987) have reported similar findings. Gaur (1980) reported that decreased nodulation and N_2 fixation in leguminous plants were a result of herbicide effects on the host plant. Audus (1964) showed that herbicides can affect formed nodules by decreasing their N_2 fixing efficiency. The application of pre-emergent herbicides may also exert effects on soil organisms (Greaves and Malkomes 1980) and on microbial mediated processes such as soil respiration, soil enzyme activity, nitrification and denitrification (Carlisle and Trevors 1986; Helweg 1986; Yeomans and Bremner 1987).

The benefits of herbicides for grass-weed control in annual pastures could be outweighed by less-obvious effects on legume production. On the other hand, the success of annual legumes in grassy pastures following herbicide application will depend on the their tolerance to different herbicides used for grass-control. Conlan *et al.* (1990) found a significant difference in the response of subterranean clover cultivars to simazine. Young *et al.* (1992) reported different responses of annual medic cultivars to a range of broadleaf herbicides.

The specific objective of this field experiment was to examine the response of a range of annual medic and subterranean clover cultivars to a number of pre-emergent herbicides, viz. Imazethapyr, Flumetsulam, Simazine, Metribuzin, Diuron and Metolachlor.

3.2.2. MATERIALS AND METHODS

3.2.2.1. Location

The experiment was conducted at the Turretfield Research Centre, 55 km north-east of Adelaide.

The experiment site had previously been sown to cereals. The soils of the area are typical of the Rosedale area, predominantly loamy red-brown earths, which are slightly acid to neutral. The area has a Mediterranean-type climate (hot dry summers and cool, wet winters). Average annual rainfall is 464 mm (84 year average 1908-1991).

3.2.2.2. Plant culture

Annual medic cultivars: *Medicago truncatula* (cvv. Caliph, Mogul, Paraggio); *Medicago polymorpha* (cv. Santigo); *Medicago scutellata* (cv. Sava) and subterranean clover cultivars: *Trifolium subterraneum* var. subterraneum (cvv. Dalkeith, Daliak, Seaton Park, Junee); *Trifolium subterraneum* var. brachycalycinum (cvv.Clare, Rosedale) and *Trifolium subterraneum* var yanninicum (cv. Trikkala), were sown on 26 June 1992. All seeds were inoculated with the appropriate inoculant (Group A (*Rhizobium meliloti*) for medics and Group C (*Rhizobium trifolii*) for subterranean clover) before sowing. The inoculant was applied as a slurry in milk. Each cultivar was sown by drill on duplicate 3 m × 30 m plots. An area of 2 m × 3 m from each plot was selected for spraying of each herbicide. Sowing rate varied between species and cultivars from 13 to 17 kg/ha depending on seed size..

3.2.2.3 Design and treatments

The herbicides used and rates of application are summarised in Table 3.2.1 and Table 3.2.3 (mixtures). All herbicides were sprayed before plant emergence using a hand-held 2m boom pressurised with CO_2 with flat fan nozzles. The experiment was of factorial design, randomised in two blocks (Diagram 3.1)

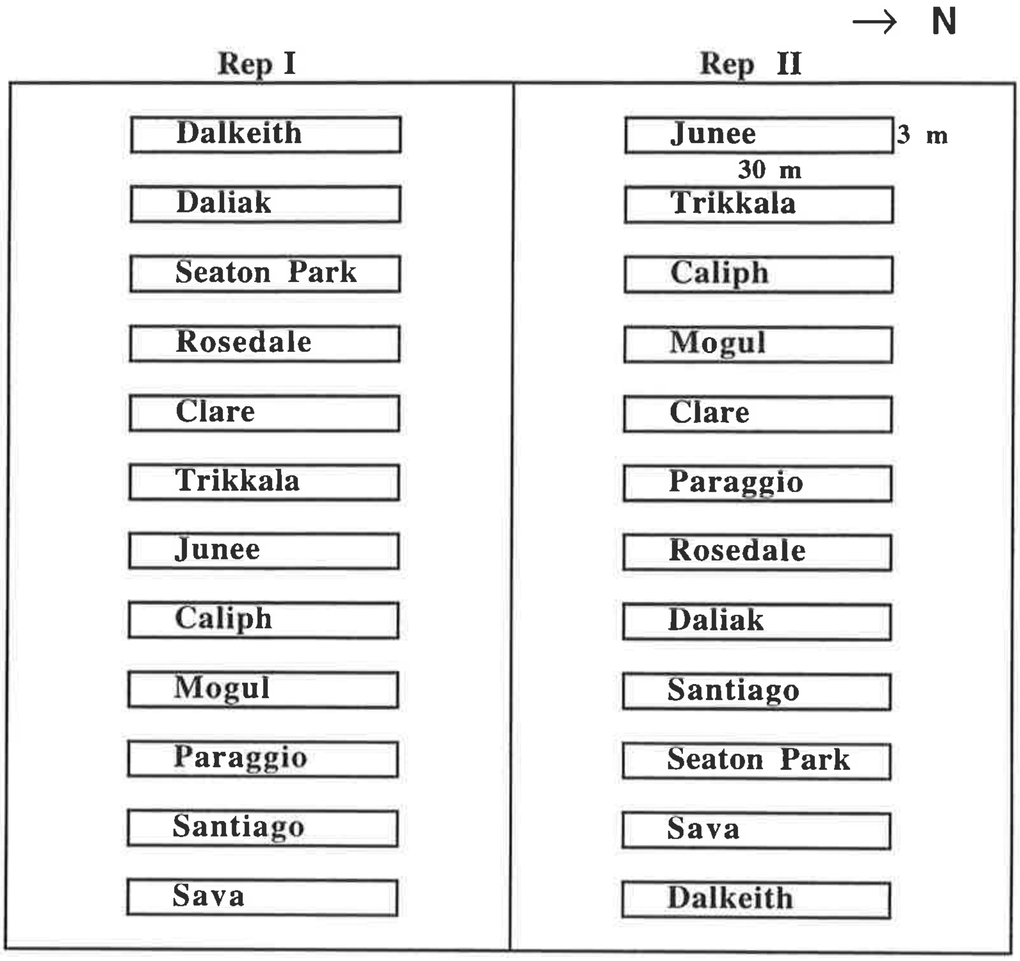


Diagram 3.1 Plan of field experiment at Turretfield Research Centre.

Table 3.2.1 Herbicides used, active ingredients and rates applied

Chemical	Rate / ha	Active ingredient (g/L)	Trade Name
Imazethapyr	200 ml	240	Pursuit
Flumetsulam	25 g	500	Broadstrike
Simazine	2.0 L	500	Lupizine
Metribuzin	300 g	750	Lexone DF
Diuron	1.5 L	500	Aguron
Metolachlor	1.5 L	720	Dual

3.2.3 DATA COLLECTION

3.2.3.1 Plant yields and nodule numbers

Ten weeks after emergence 10 legume plants from each of the experimental areas were carefully dug from the ground and soil washed from the roots. The number of nodules per plant was counted on fresh plants and dry matter determined after samples had been dried at 85 °C for 24 hours,

3.2.3.2 Statistical analyses

Statistical analysis was performed using the Super ANOVA statistical package and the interrelations between plant yield and nodule numbers were examined by means of simple linear regressions. Plant dry weight results were converted into percentages of the control to aid presentation.

3.2.4 RESULTS

3.2.4.1 General

Analyses of variance on data from all medics and clovers showed a significant interaction between cultivars and herbicides for dry matter yield and nodule numbers. The herbicide main effect was highly significant for all the cultivars of medics and clovers (Table 3.2.2).

Table 3.2.2 Summary of analyses of variance carried out on plant dry matter and nodule numbers in medic and clover cultivars

Source of variation	Medic		Clover	
	DM	Nodule No	DM	Nodule No
		(Significance levels)†		
Cultivars	***	NS	***	***
Herbicides	***	***	***	***
Cultivars × Herbicides	**	***	***	***

† NS = Not significant, ** = P<0.01, *** = P<0.001

3.2.4.2 Dry matter production

Over all medic and clover cultivars, all herbicide treatments significantly reduced dry matter production in both clovers and medics but the effect was more severe in clovers than in the medics when Imazethapyr, Simazine, Diuron, Metolachlor and Imazethapyr+Diuron were used (Table 3.2.3).

Table 3.2.3 Overall effects of 10 herbicides and herbicide combinations on dry matter reduction of medics and subterranean clovers.

Herbicides and rate of product/ha	Dry matter reduction (%)	
	Medics	Subterranean clovers
Imazethapyr 200ml	13.6	20.2
Flumetsulam 25g	9.4	9.4
Simazine 2.0L	12.0	19.9
Metribuzin 300g	7.3	7.3
Diuron 1.5L	6.8	21.5
Metolachlor 1.5L	14.0	28.2
Metolachlor 1.0L + Diuron 1.5L	9.7	7.9
Imazethapyr 150ml + Diuron 1.0L	12.3	36.4
Imazethapyr 150ml + Simazine 1.0L	7.4	5.1
Imazethapyr 150ml + Metribuzin 180g	6.3	9.0
LSD (5%)	4.2	

Varied results were obtained for the effects of herbicides on dry matter yield of cultivars (Figure 3.2.1). In medics, dry weight of Sava was higher than other cultivars at the time of the sampling but was significantly reduced ($P< 0.05$) by all the herbicides. In contrast, dry weight of Mogul was significantly ($P< 0.05$) reduced by Simazine and Metolachlor only. Differences in the efficiency of the herbicides were also observed. While Metolachlor 1.5L and Simazine 2.0 L significantly reduced DM in all cultivars, Mertibuzin 300g, Diuron 1.5L, Imazethapyr 150ml + Metribuzin 180g significantly reduced dry matter production in Sava and Paraggio only.

The percentage dry matter reduction in medics following herbicide applications differed among the cultivars (Table 3.2.4). The greatest reduction (26%) resulted from the Imazethapyr 150 ml + Diuron 1.0L application on Santiago, while the lowest value (2%) was recorded in Paraggio and Caliph by Metribuzin 300 g and Imazethapyr 150ml + Diuron 1.0L respectively.

Table 3.2.4 Effects of 10 herbicides and herbicide combinations on percentage dry matter reduction of medic cultivars.

Herbicides and rate of product/ha	Caliph	Mogul	Paraggio	Santiago	Sava
Imazethapyr 200ml	-4	4	16	20	24
Flumetsulam 25g	8	3	7	17	12
Simazine 2.0L	15	11	15	8	12
Metribuzin 300g	5	6	2	11	13
Diuron 1.5L	5	3	7	7	12
Metolachlor 1.5L	17	9	12	24	8
Metolachlor 1.0L + Diuron 1.5L	4	6	5	18	14
Imazethapyr 150ml + Diuron 1.0L	2	5	18	26	11
Imazethapyr 150ml + Simazine 1.0L	10	2	5	11	10
Imazethapyr 150ml + Metribuzin 180g	7	4	6	4	10
LSD (5%)	11	9	9	12	7

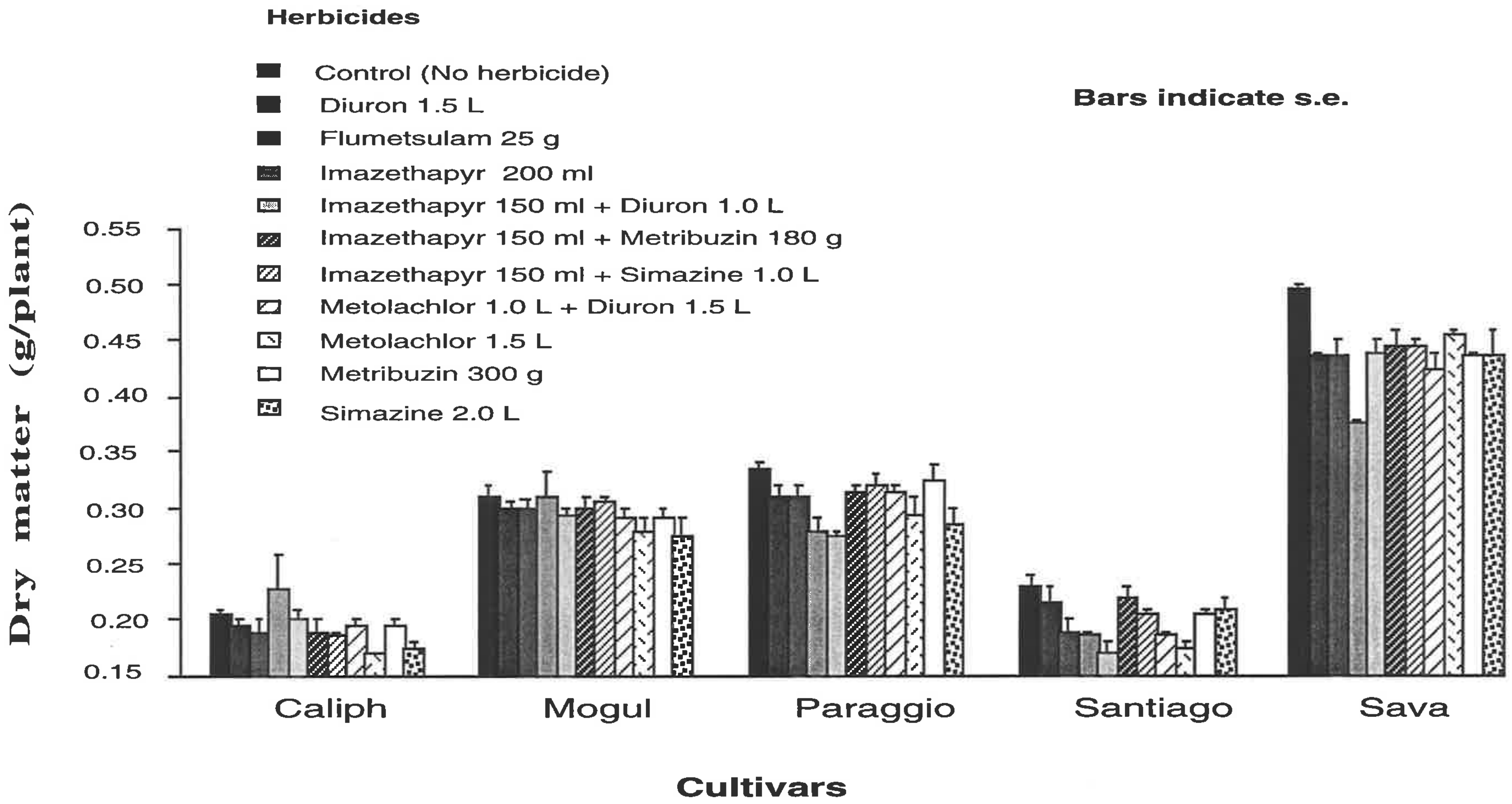


Figure 3.2.1 Effects of 10 herbicide treatments on dry matter yield of medic cultivars.

The effects of herbicides on dry weight of clovers also differed between cultivars (Figure 3.2.2). Dry matter was significantly reduced in Junee by all the herbicides except Imazethapyr+Simazine, while in Clare, dry matter production was significantly reduced by Imazethapyr+Diuron and Metolachlor and Simazine only. Due to the range of the herbicides used, the herbicides Imazethapyr, Metolachlor, Simazine and combinations of Imazethapyr + Diuron significantly reduced dry matter yield of all of the clover cultivars. In contrast, the herbicides Imazethapyr+Simazine had the least effect on the clover cultivars and its application did not significantly reduce dry matter yield of clovers except in Daliak.

Among the clover cultivars the greatest percentage dry matter reduction was recorded in Junee after the application of Imazethapyr+Diuron (65%), while the lowest was obtained in Trikkala by the herbicide Metribuzin and Imazethapyr+Metribuzin (Table 3.2.5).

Table 3.2.5 Effects of 10 herbicides and herbicide combinations on percentage dry matter reduction of subterranean clover cultivars

Herbicides and rate of product/ha	Dalkeith	Daliak	Seaton Park	Rosedale	Clare	Trikkala	Junee
Imazethapyr 200ml	20	18	12	13	12	19	47
Flumetsulam 25g	6	17	10	10	5	7	11
Simazine 2.0L	12	34	13	27	20	7	26
Metribuzin 300g	12	14	1	6	7	0	14
Diuron 1.5L	16	8	22	44	12	22	26
Metolachlor 1.5L	31	34	14	32	39	19	29
Metolachlor 1.0L + Diuron 1.5L	8	14	11	5	2	8	8
Imazethapyr 150ml + Diuron 1.0L	41	46	18	37	21	28	65
Imazethapyr 150ml + Simazine 1.0L	4	14	2	8	2	3	4
Imazethapyr 150ml + Metribuzin 180g	12	6	3	19	9	0	14
LSD (5%)	9	18	18	12	13	14	5

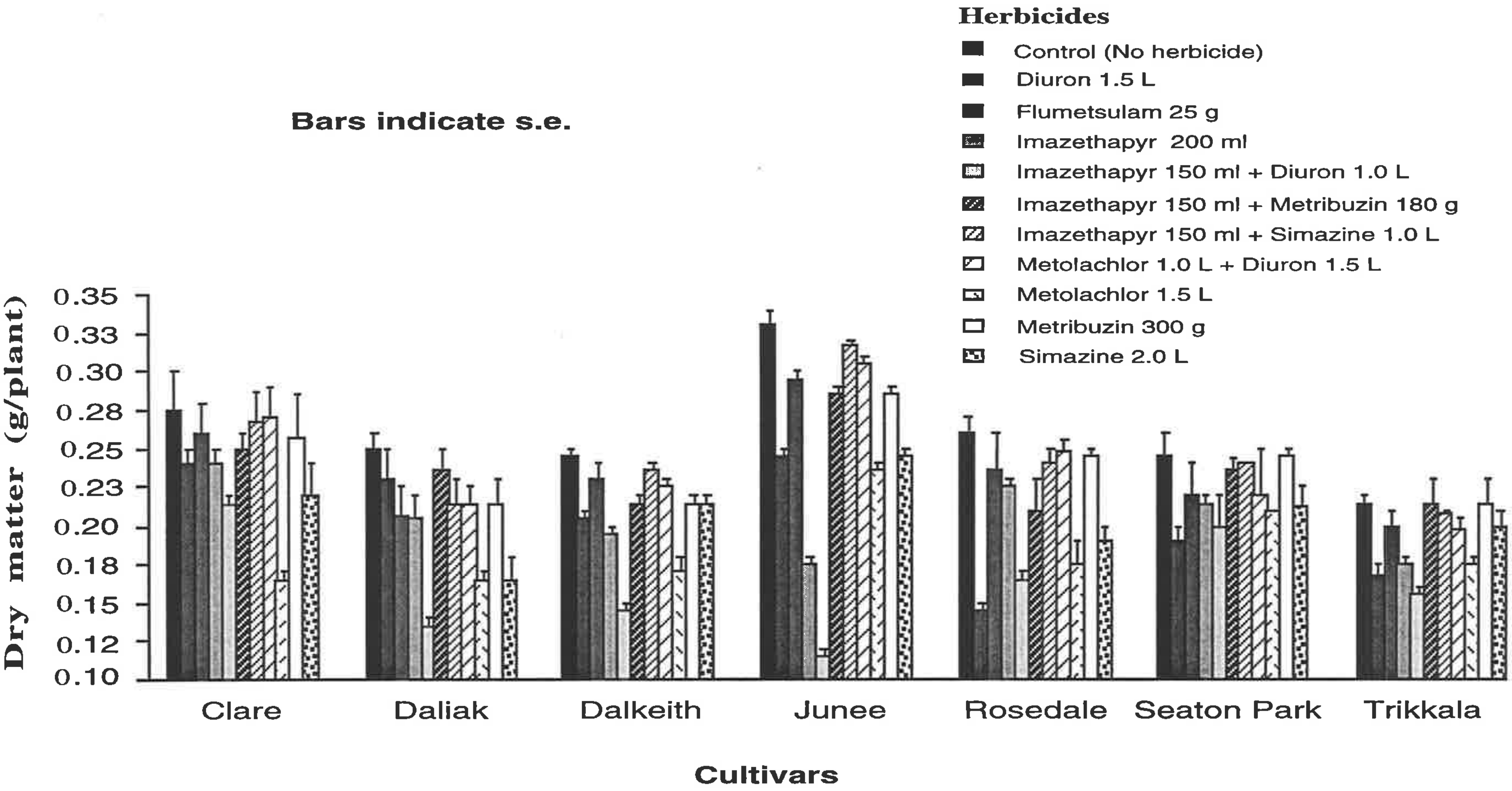


Figure 3.2.2 **Effects of 10 herbicide treatments on yield of subterranean clover cultivars.**

3.2.4.3 Nodule Numbers

Analyses of variance (Table 3.2.2) showed that the effect of herbicide on nodule numbers in medic cultivars was not significant, while the effect was highly significant for clover cultivars. The Cultivar × Herbicide interaction was also significant for both legume genera.

The herbicides affected nodule numbers in medics and clovers differently, but the inhibitory effects were more severe in medics than in clovers (Table 3.2.6). All herbicides caused significant ($P<0.05$) reduction in nodule numbers in medics. In clovers, however, the reductions occurred only when Imazethapyr 150ml + Metribuzin 180g, Imazethapyr 150ml + Simazine 1.0L and Simazine 2.0L were sprayed. The herbicides also differed in their relative effects on medics versus clovers (Table 3.2.6).

Table 3.2.6 Overall effects of 10 herbicides and herbicide combinations on the nodule numbers of medics and subterranean clovers.

Herbicides and rates of product/ha	Nodule numbers	
	Medics	Subterranean clovers
Control	133	121
Imazethapyr 200ml	100	120
Flumetsulam 25g	92	113
Simazine 2.0L	72	109
Metribuzin 300g	79	125
Diuron 1.5L	86	118
Metolachlor 1.5L	80	127
Metolachlor 1.0L + Diuron 1.5L	95	127
Imazethapyr 150ml + Diuron 1.0L	91	129
Imazethapyr 150ml + Simazine 1.0L	96	110
Imazethapyr 150ml + Metribuzin 180g	103	102
LSD (5%)	11	

Nodule number per plant in medics (Figure 3.2.3) was significantly ($P<0.05$) reduced in Caliph, Mogul and Paraggio by all the herbicides. The combination of

Imazethapyr+Simazine did not affect Sava nudoles/plant, while Santiago was more tolerant of the herbicides and nudoles/plant were not affected by the herbicides Imazethapyr, Imazethapyr+Diuron, Metolachlor + Diuron and Metribuzin.

In the clovers, nodule numbers on Clare and Rosedale were not significantly affected by any of the herbicide applications. However, in Seaton Park a reduction in nodule numbers was obtained with two herbicide combinations (Imazethapyr + Metribuzin and Imazethapyr + Simazine) while Dalkeith was most sensitive to the herbicides and showed reduced nodule numbers when sprayed with any of the herbicides with exception of the Imazethapyr 150ml + Diuron, Imazethapyr + Metribuzin and Imazethapyr + Simazine (Figure 3.2.4).

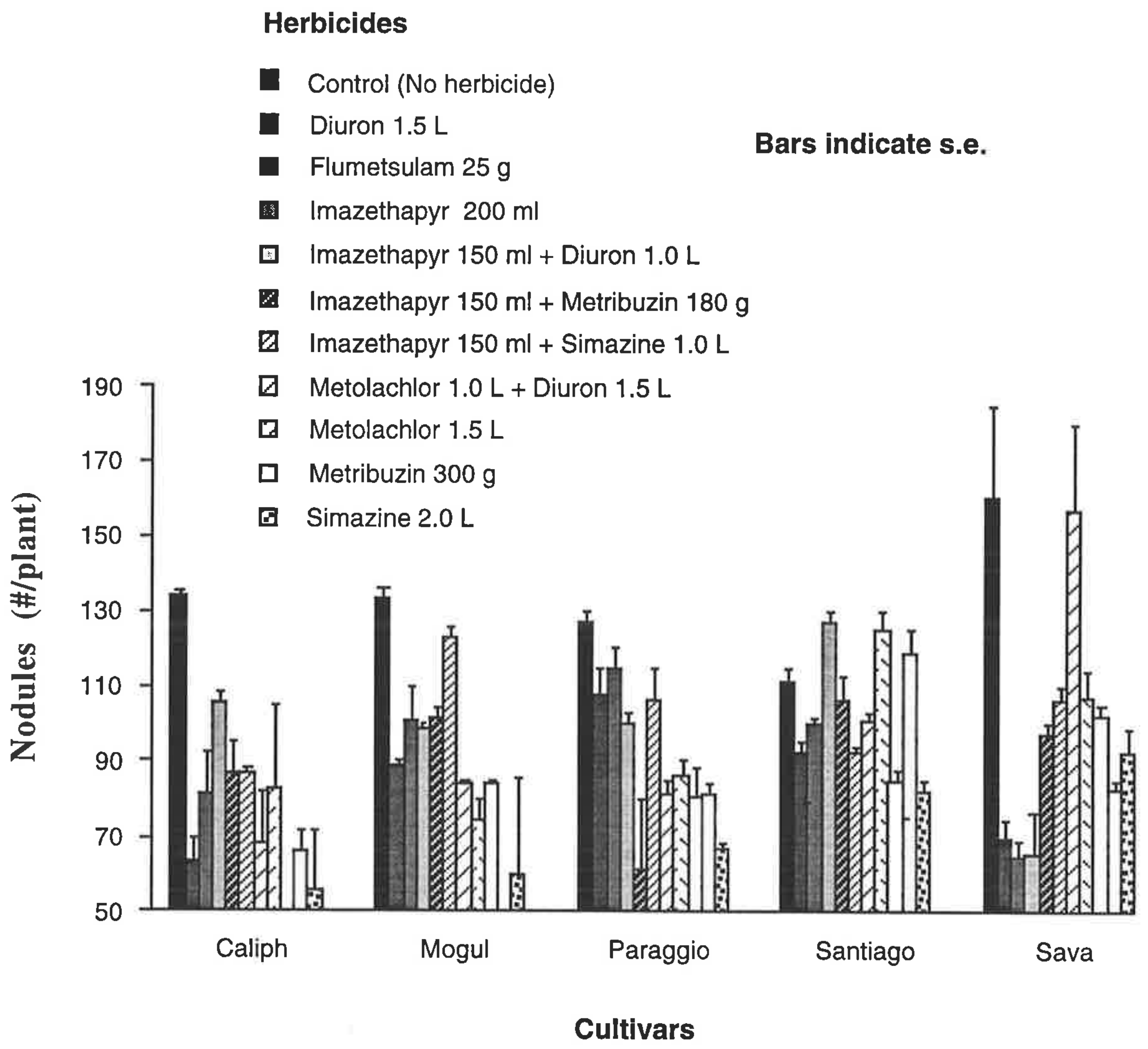


Figure 3.2.3 **Effects of 10 herbicide treatments on nodule numbers of medic cultivars.**

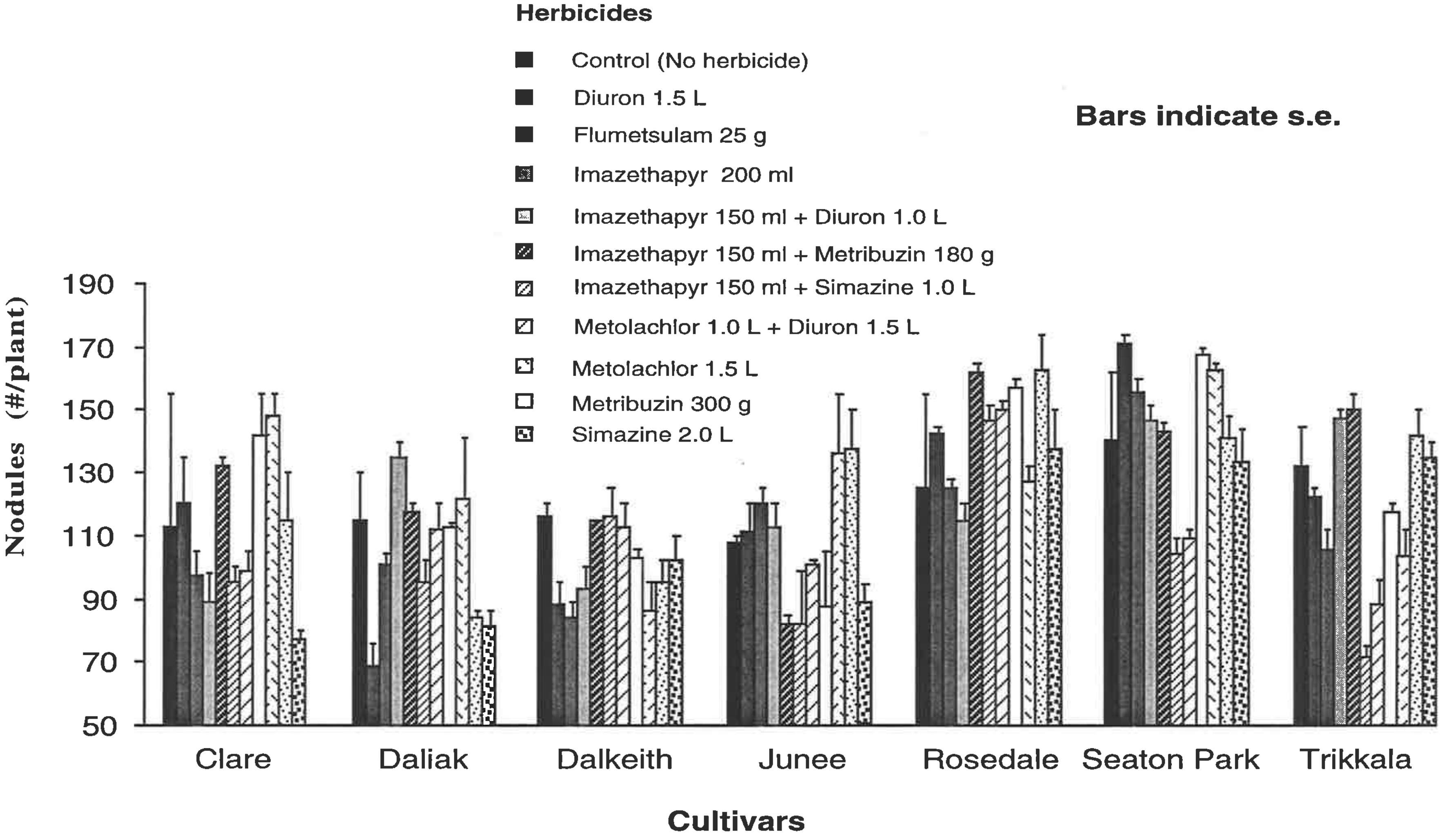


Figure 3.2.4 Effects of 10 herbicide treatments on nodule numbers of subterranean clover cultivars

The relationship between total legume plant yield and nodule numbers over all cultivars treated by the herbicides was significant in medics, but not in the sub clovers (Figure 3.2.5).

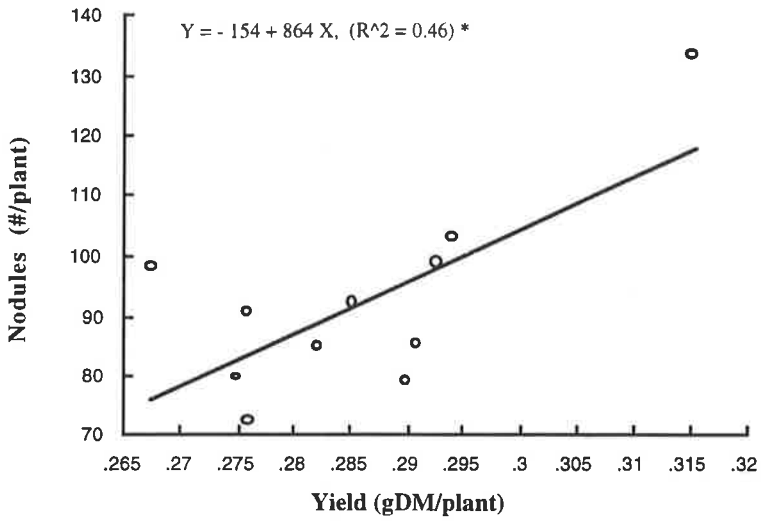


Figure 3.2.5 The correlation between plant dry matter yields (shoots+roots) and nodule numbers in medics.

Plant density was not measured, but visual observation suggested that plant density was reduced by herbicide application in most treated plots.

3.3 DISCUSSION OF BOTH EXPERIMENTS

The results show that the herbicide 2,4-DB, which was used in the Experiment 1 caused various degrees of reduction of dry matter production, nodulation and nitrogenase activity in medics. The range of herbicides used in Experiment 2, namely Imazethapyr, Flumetsulam, Simazine, Metribuzin, Diuron and Metolachlor, also affected differently the dry matter and nodule numbers of the pasture legumes. Differential effects of the post-emergent, broad-leaf herbicide 2,4-DB on several species of annual legumes have also been demonstrated by Mulholland *et al.* (1989) and Young *et al.* (1992) who showed that 2,4-DB at 2.8 and 3.5 L/ha sprayed at the 5th trifoliate leaf stage on annual medics reduced dry matter yields of SAD 2356 (*M. aculeata*) and cutleaf medic (*M. laciniata*) by up to 52%. *M.truncatula* and to a lesser extent *M. littoralis* showed considerable tolerance to this herbicide. Mulholland *et al.* (1989) also found Sephi and Harbinger to be quite tolerant of 2,4-DB, but the yield of *M. polymorpha* cv. Circle Valley was reduced by about 58%. Visual assessment showed that 2,4-DB can damage annual medic through wilting and death of petioles and leaflets, as well as reducing herbage yields. However, Mulholland *et al.* (1989) reported no leaf damage on a number of subterranean clover cultivars by a broadleaf herbicide but herbage yields were reduced by as much as 51%.

The decrease in subterranean clover yields by Simazine application in Experiment 2 was comparable with the results of Leys and Plater (1993) where the Simazine significantly reduced subterranean clover yield. Dear *et al.* (1993) have also reported differences between subterranean clovers in their tolerance to Simazine. None of the cultivars in the second experiment of the present study showed tolerance to Simazine although nodule numbers in simazine-sprayed subterranean clover cultivars namely Clare, Rosedale Seaton Park and Trikkala were unchanged when compared to the unsprayed control (Figure 3.2.4).

Differences in tolerance of subterranean clover cultivars to selective herbicides was reported by Evans *et al.* (1989). In the present study (Experiment 2), yields of Trikkala were reduced by 5 of the 10 herbicides used (Figure 3.2.2). However, Hill (1986) suggested that the tolerance of Trikkala to herbicides be ascribed to its agronomic superiority.

In Experiment 2, the data from pre-emergent application of the herbicides on legume production showed that Imazethapyr reduced dry matter in most of the cultivars, yet a report on the use of pre-emergence application of Imazethapyr to control the weed *Emex australis* in annual legume pasture (Panetta and Randall 1993), indicated that Imazethapyr treatment, in particular pre-emergence application, increased legume production and did not cause significant legume mortality in the pasture.

Fedtke (1982) reported that the mode of action of hormone-based herbicides occurred in three distinct stages, the first was a stimulation of metabolic processes including photosynthesis, respiration and nucleic acid biosynthesis, the second was stem elongation accompanied by abnormal effects such as epinasty and tissue swelling and, finally, cell membrane damage and collapse. According to Sanders and Pallett (1987) not all of these processes are necessarily affected and plants vary in response according to species and age.

Overall, the results indicated that 2,4-DB severely retarded nodule development in the upper root zone, and again the extent of the reduction differed between cultivars. However, the number of nodules on the lower half of the roots of treated plants did not differ significantly between cultivars and species. This suggests that plants have the ability to recover from the effects of the herbicide with time. In these two experiments the herbicides probably had an effect on symbiotic structures causing both nodule numbers and AR to decline. However, the work of Garcia and Jordan (1969), which showed rhizobia could grow in herbicide-treated culture and could degrade 2,4-DB, suggests that at least for 2,4-DB this was not so. The effect of the herbicide on metabolic processes of the plants was probably responsible for the effects on nodule number and AR.

Eberbach and Douglas (1989) reported that 2,4- D, which according to Alexander (1965) is an intermediate product of 2,4-DB degradation by soil micro organisms, caused decreases in nodule number per plant, when three-day-old seedlings of *Trifolium subterraneum* were treated with a range of herbicide concentrations. However, estimates for concentrations used to control weeds in commercial fields suggested that the herbicides at these levels would cause little damage to legume nodulation. They also observed that the herbicide did not affect rhizobial growth in nutrient broth. These findings support the view that the effects of the herbicide on medic-*Rhizobium* symbiosis may be mediated through effects on the host plant and not the *Rhizobium per se.* Eberbach and Douglas (1989) again observed that when inoculum prepared in a 2,4-D-treated broth was centrifuged and washed, the small amount of herbicide carried over with the *Rhizobium* was enough to stimulate nodulation and probably root growth. Jordan and Garcia (1969) also observed a mild stimulation of growth of *Lotus corniculatus* rhizobia by lower levels of 2,4-DB. They found that the inhibitory effects of higher levels of 2,4-DB were not permanent. No inhibition of host plant nodulation was observed with cells of *Lotus* rhizobia pre-incubated with 2,4-DB at concentrations up to 10 μg/ml. In fact the number of nodules on plants inoculated with cultures grown at low herbicide concentrations appeared to be higher than on controls. This suggests that medic plants sprayed with the herbicide may even be stimulated to grow more rapidly when the concentrations have been reduced to very low levels with time. This was observed in Experiment 1, where 24 days after early spraying Parabinga, Harbinger, Harbinger (AR), Kelson, Sava and Paraponto plants sprayed at half the recommended rate of the herbicide had more lower nodules than the controls. Fedtke (1982) also reported that sublethal applications of auxin analogue herbicides may result in stimulation of biochemical pathways and also perturbation of growth.

The results of Experiment 1 at the Waite Institute also provide some evidence which suggests that the effects of the herbicide 2,4-DB on the medic plants are temporary, differ between cultivars and species, and may mainly be effects on the host plant. This is also in agreement with the findings of Wache (1987) who reported only temporary harmful effects of the herbicide 2,4-DB on the symbiotic properties of *M. sativa.*

The data from Experiment 1, however, indicated that A R followed the same trend as nodule number or growth rate. Overall, 2,4-DB spray significantly reduced total dry weight, total nodule number, and AR activity at both rate and time of application. The AR activity of control legumes differed significantly from those legumes sprayed with the recommended and half the recommended rate of herbicide application and there was a significant interaction between the herbicide and cultivar for AR activity. Genotypes responded differently to the herbicide, the greatest reduction in AR activity obtained in Kelson (88%) and least reduction (12%) recorded for Harbinger.

In the first experiment, overall nodule numbers in the cultivars correlated with dry matter production and AR activity after the late application of a post-emergent herbicide and did not correlate after the early application. Although there is a positive and significant correlation between nodule number and dry matter production, the correlation coefficient is low, indicating that only a small proportion of variation in nodule number is explained by dry matter production (Figure 3.1.7). Also, possibly the growth rate was rapid and new nodules were formed at an early stage of plant growth after early application of the herbicide. It is concluded that in some situations herbicides may influence the legume-*Rhizobium* symbiosis by affecting bacterial numbers without affecting the legume (Eberbach and Douglas (1989). An example from the data of Torstensson (1975) is that in white clover treated with the herbicide bentazon a reduction in plant dry weight correlated with that of AR activity, while for field bean treated with bentazon a 40% reduction was observed in AR activity without any reduction in plant dry weights. In the second experiment in this Chapter, in medic treated with a range of herbicides, the correlation between nodule number and plant dry weight was significant, but this was not apparent for sub clover cultivars. However, the observed effects of herbicides differed from cultivar to cultivar in both experiments.

3.4 CONCLUSIONS.

The experiments described in this chapter have shown that 2,4-DB herbicide often significantly reduces dry matter production, nodulation and N_2 fixation of medics. The effects appear to be temporary and differ between cultivars. Cultivars that tolerate the effects of the herbicide can be selected.

Time of application of the herbicide appears to be very important for the recovery of plants sprayed with 2,4-DB. Plants sprayed early have the capacity to recover so that the effects of the herbicide on nodulation and N_2 fixation is reduced. Late applications appear to be more detrimental to plants.

Pre-emergent herbicides (6 pure and 4 mixtures of these herbicides) generally reduced dry matter production, nodulation and N_2 fixation of a range of medics and sub clovers.

Evaluation of the effects of these herbicides shows that leguminous plants can be damaged by many herbicides, either prior to nodule formation, during nodule formation or in the nodulated stage. Therefore, management practices which involve the use of herbicides that are not deleterious to legumes, can reduce potentially-detrimental effects on pasture legumes to ensure the maintenance of N inputs via nitrogen fixation and thereby improve pasture production and soil fertility.

CHAPTER 4

4.1 EXPERIMENT 3. THE EFFECTS OF SIMAZINE MIXTURES APPLIED AT DIFFERENT RATES AND GROWTH STAGES ON THE YIELD AND NITROGEN FIXATION OF SUBTERRANEAN CLOVER IN GRASSY PASTURE. (Location: Field at Kapunda).

4.1.1 INTRODUCTION

Subterranean clover (*Trifolium subterraneum*) is the most important annual pasture legume in southern Australia because of its high forage production and its potential for nitrogen fixation (McIvor and Smith 1973). It tends to decline in annual pastures after initial establishment through invasion by vigorous annual grasses such as barley grass (*Hordeum leporinum*) and brome grass (*Bromus* spp.) and other nitrophylous weeds. Barley grass tends to replace subterranean clover by successfully competing against the clover (Rossiter 1966) following nitrogen fixation. However, in addition to soil N levels, the stocking rate or short-term grazing pressure can influence both pasture yield and botanical composition (Carter 1990a). Since legume-dominant pastures are desirable to replace the soil N removed by cereal crops and to reduce the incidence of cereal diseases, the competition between grass and clover in a temperate pasture is of great agricultural significance particularly where there is a reliance on symbiotic nitrogen fixation for maintenance of adequate soil N levels (Butler 1987).

Barley grass in the vegetative stage is a useful grazing plant, but later, at the mature stage, awned seeds injure livestock. These seeds cause eye damage which significantly reduces growth rate of grazing lambs and can also cause skin damage during grazing (Atkinson and Hartley 1972; Little *et al* .1992,1993). Thus grass-weed control should be an important objective to increase production and nitrogen inputs in a grass/legume pasture.

Herbicide use is probably the most common means of weed control, but in a grass/legume pasture the characteristics of the legume and grass components must be considered in the selection, application and placement of the herbicide (Combellack 1989). In the search for a suitable herbicide for control of barley grass in a subterranean clover pasture, there are many questions about the side effects of the herbicides on the growth and nitrogen fixation of the subterranean clover component, and there are other problems to consider, such as paraquat resistance of barley grass in pastures (Powles 1986).

A selective herbicide at a minimum rate which achieves a satisfactory level of weed control, which optimises economic returns to the user and has minimal adverse effects on the environment, should be considered (Combellack 1990). The correct timing of herbicide application is also important: for example, removal of weeds, while still small, from pastures allows the use of a lower dose of herbicide and invariably leads to better final pasture yields. Under most circumstances, late-emerging weeds are few in number and not as deleterious as those emerging earlier (Combellack 1992). Generally, non-selective herbicides e.g. Gramoxone® may remove weeds from pastures, but damage the legume component of the sward (Thorn and Perry 1983; Leys and Plater 1993).

Although Fusilade® (active ingredient fluazifop-P) is a very effective grass-selective herbicide for controlling most grasses and improving the yield and seed production of subterranean clover (Carter 1990b), it does not control silver grass at normal rates and also it is expensive. Therefore, simazine is commonly used to control silver grass in the wetter parts of the cereal zone and higher-rainfall areas of southern Australia.

For the above reason, an experiment was conducted at a field site near Kapunda, South Australia, to evaluate the effects of some commonly-used herbicide combinations on yield and N_2 fixation of subterranean clover in a pasture, using combinations of simazine+paraquat and simazine+fluazifop-P at different rates and times of application. The use of wetting agent was also examined. The ^{15}N isotope dilution technique (using the ^{15}N naturally present in soil) was used to measure nitrogen fixation by the legume.

This study aims to assess the efficacy of these herbicides in controlling grasses and their effects on nitrogen fixation and dry matter yield of subterranean clover with the objective of optimising herbicide use in pasture management.

4.1.2 MATERIALS AND METHODS

4.1.2.1 Site of the experiment

The experiment was carried out at the CSIRO field experiment area on a commercial farm near Kapunda, South Australia. Choice of the area was based on paddock history and soil uniformity. The area had a grass/clover pasture (c. 40% clover and 60% grasses) for approximately 6 years. The soil at the experiment site was a red-brown earth, classification Dr 2.33 (Dudal 1970), with a pH in water of 6.0. The climate is Mediterranean-type with hot, dry summers and a winter-dominant rainfall pattern. Average annual rainfall in this district is 450 mm.

4.1.2.2 Pasture

The site was covered by an old stand of *Trifolium subterraneum* (hereafter referred to as subterranean clover or sub clover), some grasses and a variety of broad-leaved weeds. The predominant cultivars of sub clover present were Clare and Mt Barker, while the grass weeds included barley grass (*Hordeum* spp.), brome grasses (*Bromus* spp.), ryegrass (*Lolium rigidum*), silver grass (*Vulpia* spp) and wild oats (*Avena fatua).* The major broad leaf weeds were cape weed (*Arctotheca calendula*), geranium (*Erodium botrys*), Salvation Jane (*Echium plantagineum*) and soursob (*Oxalis pes-caprae*) .

The experimental area was continuously grazed by sheep throughout the remainder of the year along with the surrounding area of the paddock in which the trial was situated.

4.1.2.3 Experimental design and treatments

The experiment was a randomised complete block design (Plan 4.1) superimposed on an existing pasture in 1992 (Plate 4.1). Plot sizes were 2 m $\times$ 30 m replicated 4 times.

Plate 4.1 A general overview of the Kapunda plots showing the impact of herbicide application.

The treatments involved the following chemicals: (i) Simazine at 0.75 and 0.9 kg/ha active ingredient; (ii) Fusilade 212 at 0.5 kg/ha (active ingredient fluazifop-P); (iii) Gramoxone at 0.25 and 0.3 kg/ha (active ingredient paraquat).

The herbicide mixtures were applied to plots with a small, conventional boom spray, fitted with Tee Jet 600, 1 LP nozzle, adjusted to 50 cm above the plants. (Inwood, pers. comm). Agral 600 was used as wetting agent in all spray mixtures except one using Simazine+paraquat. The treatments applied are shown on Plan 4.1.

4.1.3 DATA COLLECTION

4.1.3.1 Shoot samples

One quadrat sample (50 × 50 cm) was cut to ground level from each plot (44 quadrats from 11 treatments replicated 4 times), on 25 September i.e. 1 to 3 months after herbicide application (depending on date of spraying), the quadrats were close to a transect line running from east to west across the plots. Areas with a high density of capeweed were avoided.

Plan 4.1 Field layout for Exp. 3

Complete block design

Plots being 2 m X 30 m

Treatments applied on dates shown:

1 Control (No herbicide)

2 Simazine+Paraquat
Rate 0.9+0.3 kg a.i.† /ha
Date 25/6/92

3 Simazine+paraquat (no wetter)
Rate 0.9+0.3 kg a. i./ha
Date 25/6/92

4 Simazine+paraquat
Rate 0.75+0.25 kg a. i./ha
Date 25/6/92

5 Simazine+fluazifop-p
Rate 0.9+0.5 kg a. i./ha
Date 25/6/92

6 Simazine+fluazifop-p
Rate 0.9+0.5 kg a. i./ha
Date 9/7/92

7 Simazine+paraquat
Rate 0.9+0.3 kg a. i./ha
Date 9/7/92

8 Simazine+paraquat
Rate 0.9+0.3 kg a. i./ha
Date24/7/92

9 Simazine+paraquat
Rate 0.9+0.3 kg a. i./ha
Date 6/8/92

10 Simazine+paraquat
Rate 0.9+0.3 kg a. i./ha
Date 24/8/92

11 Control (No herbicide)

→N

I	II	III	IV
1	3	11	1
2	4	6	8
3	1	2	10
4	4	3	11
5	7	8	6
6	8	9	9
7	5	10	4
8	6	7	7
9	10	5	2
10	9	4	3
11	11	1	5

† a. i. = active ingredient

All plants within each quadrat were cut to ground level with a knife, bulked, placed in labelled bags and taken to the laboratory. The bulked samples were then hand- separated into clover, grass, and other species, washed free of soil and placed in a forced-draught dehydrator, at 85 °C for 24 hours for dry matter estimation and sub-samples at 60 °C for 24 hours for nitrogen fixation measurements. Dry weights were recorded and the sub-samples milled to a fine uniform powder with a Lab Technics model L M-ring roller bowls for ^{15}N measurement . The rings and bowls were washed with warm water and rinsed with 95% alcohol after grinding of each sample to prevent cross contamination. The finely-ground clover and grass samples were subsequently used to determine total N and ^{15}N contents by mass spectrometry.

4.1.3.2 Root samples

Two soil cores (14.7 cm diameter and 30 cm depth, total of 88 soil cores) were taken from each harvested quadrat site immediately after herbage sampling to obtain clover and grass root samples in the experimental area. A truck-mounted Fletcher hydraulic driven rig was used for the soil coring. The cores were placed in labelled plastic bags and taken to the laboratory. Each soil core was then transferred to a 2mm wire mesh container and washed under a tap until all roots were washed free of soil: the root material was separated into clover, grass and mixed roots. Clover plant densities were determined by counting the subterranean clover tap roots complete with crown in all 88 cores.

Nodule score was obtained from the number of nodules on the root system as described by Corbin *et al.* (1977). For each soil core the scores for all plants were added together and divided by the number of plants to obtain a mean nodule score. A score of 0 - 2 represents poor nodulation; 2 - 3 represents fair nodulation; 3 - 4 represents good nodulation; 4 - 5 represents excellent nodulation.

The clover and grass root samples were subsequently dried at 60 °C for 24 hours for dry matter measurement and N and ^{15}N determination as described above for plant top material.

4.1.3.3 Soil samples

Soil cores were taken to determine the variation of ^{15}N level, total nitrogen and inorganic nitrogen at the site. The cores were taken from different areas (from the corners and from middle of the experimental area), the cores were 5 cm diameter by 30 cm depth, (split into 0-5, 5-10, 10-20, 20-30 cm depths). Moist soil samples were placed in labelled plastic bags and taken to the laboratory where the soil samples were air dried at low temperature (room temperature) in an NH_4-free environment. The dried soil samples were then finely ground. Each soil sample was oven dried at 80 °C. Samples of about 45 mg (total of 75 samples 3 replicates from each core and depth) were weighed accurately into tin capsules and run on the mass spectrometer to determine the variation in the soil $\delta^{15}N$ and total N levels in the soil cores taken over the experimental area.

Inorganic N was measured as follows: A sample of 50 g (total of 100 samples 4 replicates from each core and depth) of the sieved dry soil was shaken with 125 ml of 2 M KCl (149 g KCl dissolved in 8 litre NH_4-free double- distilled water) for 1 hour in stoppered flasks, the resulting slurry was centrifuged and the supernatant passed through Whatman No.42 filter paper. The extracts were loaded into the rack on the sampling deck and analysed on the multi-channel auto-analyser. Nitrogen was read directly from the auto-analyser chart in mg NH_4-N/L or mg NO_3-N/L and a computer program was used to calculate the concentration in the soil.

4.1.3.4 Potential reference plant samples

Shoot samples of the associated weeds that could be used as non-fixing reference plants viz. barley grass, brome grass, silver grass, rye grass, wild oats, wire weed, cape weed, Salvation Jane and geranium were collected from small areas (about 1 m^2) as near as possible to control plots to determine their variability of ^{15}N and total N contents to check if different non-fixing plants take up soil available N of different ^{15}N content. This can affect the estimated proportion of N fixed (P fix - see below). The samples were prepared for ^{15}N determination as described earlier.

In addition, shoot samples of barley grass were collected from 6 different areas from north to south in each of the control plots to check the variability of $\delta^{15}N$ across the plots. The samples were then prepared for ^{15}N analysis as before.

4.1.3.5 N_2 fixation measurement

The clover and grass materials were analysed for ^{15}N by mass spectrometry. Then the natural abundance technique was used to estimate N_2 fixation integrated from emergence to harvest. The requirement of the technique is that legume and reference plant assimilate the indigenous soil N with the same ^{15}N content during growth (Ledgard *et al.* 1985a). Approximately 6 mg of finely ground sample (containing about 200 µg N) was weighed into tin capsules (five replicates, total of 400 from clover and grass shoots and total of 800 from clover and grass roots) and its ^{14}N and ^{15}N contents determined using a mass spectrometer (Europa Classic tracermass mass spectrometer stable isotope analyser). Ammonium sulphate calibrated against an IAEA ^{15}N standard was used as the reference material for the mass spectrometry. The proportion of clover nitrogen fixed from atmospheric N_2 was calculated from the following equation (Peoples *et al.* 1989).

$$\text{P fix} = 100 \frac{\delta^{15}\text{N grass} - \delta^{15}\text{N legume}}{\delta^{15}\text{N grass} - B}$$

where P fix is the proportion of clover nitrogen which originated from atmospheric N_2, $\delta^{15}N$ grass and $\delta^{15}N$ legume are the δ values of N_2-fixing subterranean clover and non-N_2-fixing barley grass respectively associated together in the pasture. The value of B is the $\delta^{15}N$ value of total N accumulated by nodulated clover growing in N-free nutrient solution in a controlled N-free environment (This will be described in the next experiment). The amount of N_2 fixed by subterranean clover was calculated by multiplying P fix by the shoot and root N content at the time of sampling.

4.1.4 RESULTS

4.1.4.1 Effects of the herbicides on yield of clover and grasses

***Type of herbicides*:** Determination of the dry matter production of plants treated with different herbicides showed that grass weed, clover and their parts responded differently to the different herbicides. Simazine+paraquat and simazine+fluazifop-P sprayed on 25/6/92 at the recommended rate decreased grass shoot and root DM relative to the unsprayed control. Analyses of variance carried out on dry matter yields, show a significant effect of species and a significant Treatment × Species interaction for plant shoot weight. The main effect of treatment on shoot and root dry matter was non-significant. This indicated a differential effect of herbicide on clover and grass dry matter yield in the legume/grass pasture and the reduction of a grass proportion led to an increase in clover proportion (Table 4.1.1).

Table 4.1.1 Summary of analyses of variance on dry matter yield of clover and grass shoots and roots (kg/ha) obtained following the herbicide application to the pasture.

		Source of variation and significance levels.		
Affecting factors	Plant parts	Treatments	Species	Treatments×Species
Herbicide treatnent				
	Shoots	NS	***	*
	Roots	NS	NS	NS
Rate of application				
	Shoots	NS	***	*
	Roots	*	**	NS
Wetting agent				
	Shoots	NS	**	NS
	Roots	NS	NS	NS
Time of spraying				
	Shoots	NS	***	*
	Roots	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

A significant (P<0.05) reduction in DM was recorded for grass shoots and roots following spraying with simazine+paraquat. But simazine+fluazifop-P significantly (P<0.05) decreased DM of grass shoots only. The reduction in grass root DM was greater in the simazine+paraquat treatment than in the simazine+fluazifop-P plots when sprayed at the same time (25/6/92) . In clover plants, shoot DM was increased, but not significantly by both herbicide mixtures. While clover root dry matter increased significantly ($P<0.05$) following simazine+paraquat treatment, no significant increases were obtained with simazine+fluazifop-P treatment. In short, clover shoot and root DM production was higher in the simazine+paraquat treatment than in the simazine+fluazifop-P treatment (Fig 4.1.1). In this study, the decrease of the grass relative to the clover demonstrated the selectivity and efficiency of the herbicides used on the grass component .

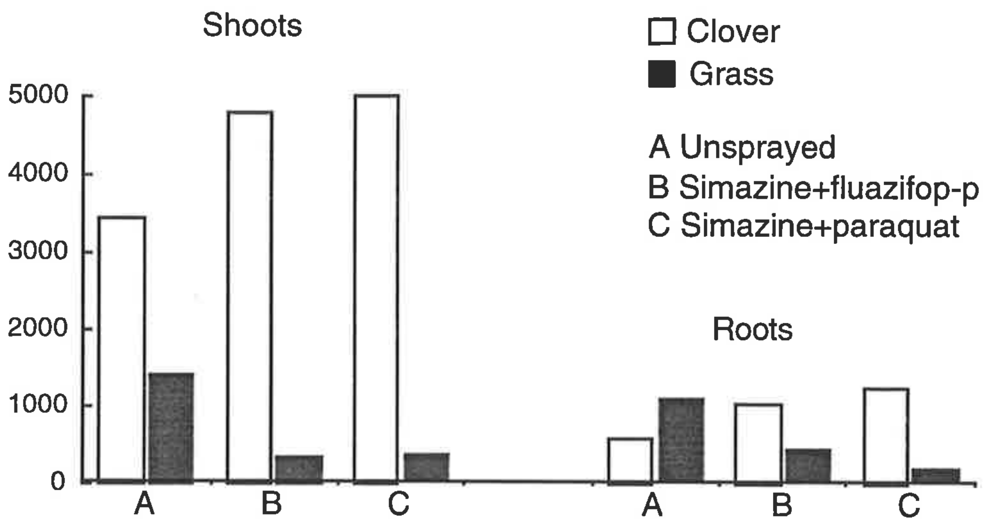


Figure 4.1.1 Effects of different types of herbicides sprayed at the same time at the recommended rate of application on clover and grass dry matter yields in pasture.

***Rate of application*:** The rate of the herbicide application rather than herbicide type could be the main factor affecting yield of the pasture. Analyses of variance on dry matter yields obtained over the experiment show a significant interaction between rate of herbicide application and legume and grass species and also highly significant effects from different plant species on shoot dry matter yield. The main effects of species and rate of application was observed on root yields. However, the Treatment×Species interaction for root yield was not significant (Table 4.1.1). When the simazine+paraquat mixture was applied at the early stage of growth at two different rates, the higher rate of simazine+paraquat (0.9+0.3 kg/ha) was more efficient in increasing the proportion of clover shoots and roots in the pasture than the lower rate (0.7+0.25 kg/ha). However, analyses of variance of dry matter production of clover sprayed by two rates of herbicide plus unsprayed control, revealed no significant effects of the herbicide at both rates on the clover shoot component of the pasture, relative to the weedy control. While clover root DM increased significantly ($P<0.05$) at the higher rate of herbicides, no significant differences were obtained at the low rate of herbicide application on clover root DM compared with that of the unsprayed control. With grass, the opposite occurred, and a yield decrease was observed with both application rates of the herbicide. Grass shoot DM was decreased significantly ($P<0.01$) by both the high rate ($P<0.05$) and by the low rate of the herbicide application when compared with unsprayed control containing weeds. The low rate of herbicide application decreased yield of the grass root DM, but the decrease was not significant, while high rate of application significantly ($P<0.05$) reduced grass root DM yield. (Figure 4.1.2).

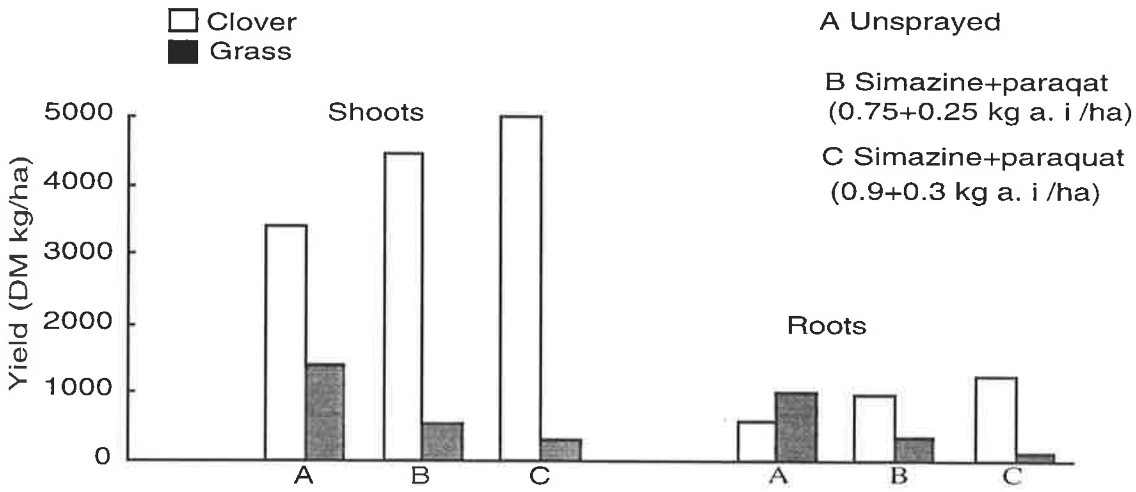


Figure 4.1.2 Effects of two rates of herbicide (sprayed at same time) on clover and grass yield .

Time of spraying: The variation in clover and grass DM yields were highly dependent on the time of herbicide application. Analyses of variance carried out on data for the different times of the herbicide application together show an interaction between time of herbicide application and legume/grass species on plant shoot DM. A highly significant effect of species also was obtained on the shoot DM, while there was no significant effect of species, treatment and their interaction on plant roots due to different herbicide applications (Table 4.1.1). Clover shoot DM yields were significantly higher ($P< 0.05$) when the simazine+paraquat was sprayed early (25/6/92 and 9/7/92) and were lowest (not significant) at the late spraying (24/8/92). Significant decreases in clover shoot DM also occurred between the early and late times of application of simazine+paraquat (Figure 4.1.3). For clover root DM, significant differences ($P<0.01$) were obtained between early-sprayed clover and the unsprayed weedy control. At other times of spraying, clover root DM was not significantly affected. There were also significant ($P< 0.05$) differences in clover root dry matter between early and later times of spraying. In contrast, the amount of grass shoot DM decreased relative to the control at all times of spraying. This decrease was highly significant ($P<0.01$) when the herbicide was sprayed early (25/6/92 and 9/7/92), significant ($P< 0.05$) at mid spraying (24/7/92), but not significantly different from the control with late applications of herbicide (6/8/92 and 24/8/92). The grass root DM also decreased after the application of herbicides. The earliest application of simazine+paraquat (25/6/92) was significantly different from the unsprayed control at $P< 0.01$, at $P< 0.05$ with application of simazine on 9/7/92 and 24/7/92 and not significantly different from the control at later application times (Figure 4.1.3).

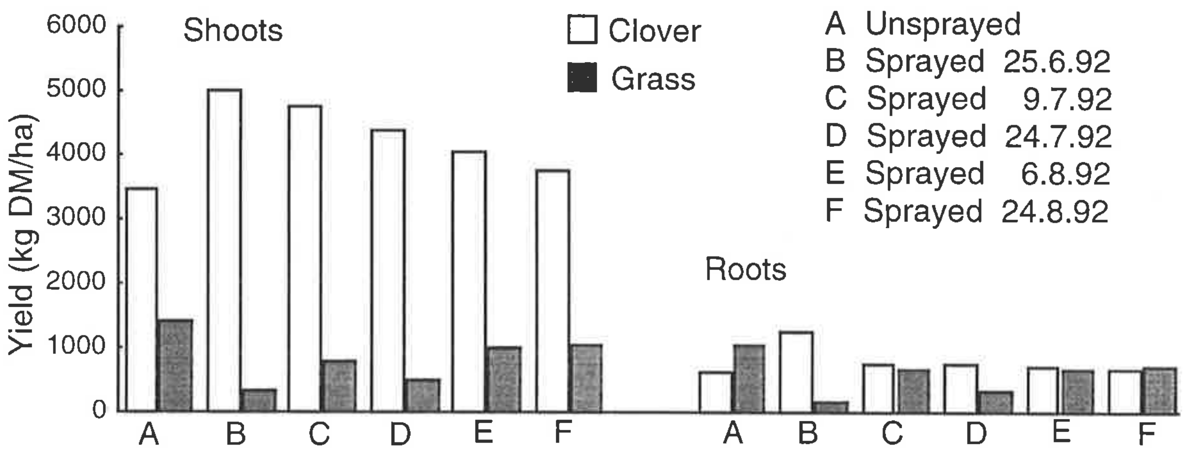


Figure 4.1.3 Effects of simazine+paraquat applications at different spraying times on clover and grass yields.

The effects of the simazine+fluazifop-P mixture on the clover DM yields showed that the proportion of clover shoot DM in the pasture was greatest (not significant) relative to the unsprayed control when the herbicide mixture was sprayed early (25/6/92) whereas the later application on 9/7/92 did not have as much effect (Table 4.1.2). However, no significant differences were obtained for clover root DM at either time of the application when compared to that of the unsprayed grassy control. The simazine+fluazifop-P mixture reduced grass shoot DM at both times of spraying (significant at $P<0.01$ for the early spraying and at $P<0.05$ for the late spraying). Grass root DM yield was also decreased by the simazine+fluazifop-P treatments at both times of spraying, but only the late spraying caused a significant decrease ($P<0.05$). In fact, greatest reductions in grass dry matter yield were obtained from simazine+fluazifop-P following the 9/7/92 application (Table 4.1.2).

Table 4.1.2 Effects of simazine+fluazifop-P mixture at different times of spraying on clover and grass yields (kgDM/ha).

Date	Clover		Grass	
of spraying	Shoot	Root	Shoot	Root
Control	3422	667	1392	1164
25/6/92	4778	1029	347	359
9/7/92	4075	580	173	199
LSD ($P<0.05$)	NS	NS	805	954

***Wetting agent*:** The herbicide treatment with addition of the wetting agent decreased grass shoots and roots significantly ($P<0.05$) compared to the unsprayed control. The DM yield of grass shoots and roots that were sprayed with herbicide without wetting agent did not differ significantly in their DM relative to the unsprayed control, probably due to better contact between plant and herbicide with wetting agent . In contrast, lower clover shoot and root DM was obtained by the herbicide alone when compared to the DM obtained with wetting agent plus herbicide: however, this reduction was not significant (Figure 4.1.4). The addition of wetting agent appeared to be an important factor influencing the efficiency of the herbicide. Despite this, the main effect of treatment and the Treatment × Species interaction were not significant for either shoot or root dry matter. The only significant difference was obtained between the shoot dry matter of the grass and clover (Table 4.1.1).

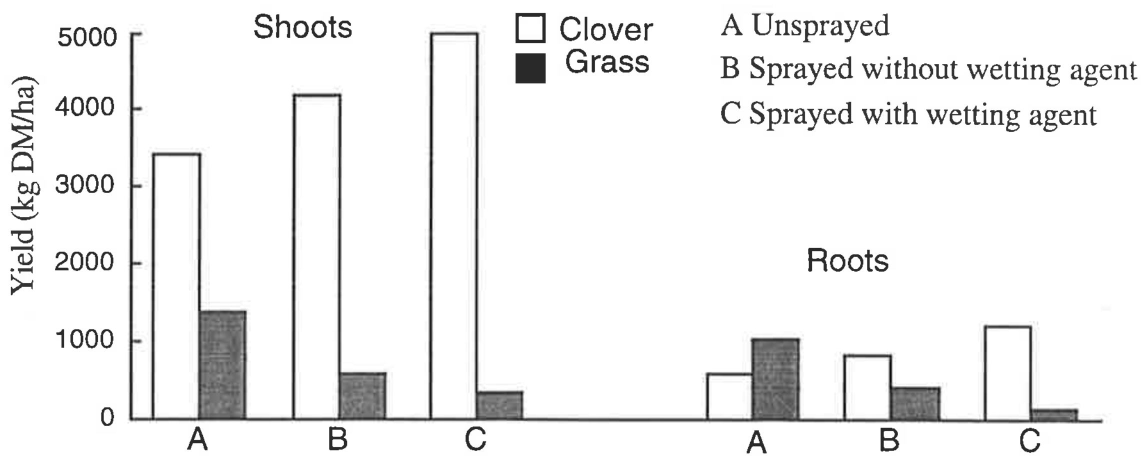


Figure 4.1.4 The response of clover and grass DM to the addition of wetting agent to a simazine+paraquat mixture.

4.1.4.2 Effects of herbicides on subterranean clover root density

There was a non significant reduction in the number of subterranean clover roots following herbicide treatments when compared with the unsprayed control. The greatest reduction in root number was obtained with simazine+ fluazifop-P applied in July (Table 4.1.3)

Table 4.1.3 Effects of time of application of herbicides on the root density and nodule number of subterranean clover.

Treatments	Date of spraying	Root number/m^2	Nodule score
Unsprayed control		7553	1.8
Simazine+paraquat	25/6/92	6116	2.8
Simazine+paraquat	9/7/92	5674	2.5
Simazine+paraquat	24/7/92	6779	2.7
Simazine+paraquat	6/8/92	6821	2.6
Simazine+paraquat	24/8/92	7074	2.3
Simazine+fluazifop-P	25/6/92	6561	2.4
Simazine+fluazifop-P	9/7/92	5526	2.3
LSD (5%)		NS	0.6

4.1.4.3 Effects of herbicides on nodule numbers

Clover nodule numbers were increased considerably by all herbicide treatments: analyses of variance revealed significant differences ($P< 0.05$) for simazine+paraquat at all times of spraying in comparison with the unsprayed control with weeds (Table 4.1.3). The simazine+fluazifop-P showed a significant increase in nodulation only for the early spraying.

Early application of the simazine+paraquat with or without wetting agent increased clover nodule numbers significantly ($P<0.05$) when compared to the unsprayed control, and there was a significant increase in clover nodule numbers when plants were sprayed with the addition of wetting agent to the herbicide (Table 4.1.4).

Both rates of simazine+paraquat (sprayed on the same date) increased nodule numbers compared to the unsprayed clover, and there were no significant differences in nodule numbers between the low rate (0.75+0.25) and the high rate (0.9+0.3) of herbicide application: however, the clover sprayed at the higher rate had higher nodule numbers (Table 4.1.4).

There was no correlation between nodule number and P fix in treated plants. The significant differences in nodule number may not be a direct effect of application of herbicide. Control of grass in the pasture may have reduced competition between sub clover and grass weeds and hence increased the nodule number on the sub clover roots.

Table 4.1.4 Effects of different herbicides and rates of application when sprayed early (25/6/92) on root density and nodule number of subterranean clover.

Treatments	Rate (kg a.i./ha)	Root density/m^2	Nodule score
Unsprayed control	-	7553	1.8
Simazine+paraquat (with wetting agent)	0.75+0.25	6853	1.9
Simazine+paraquat (with wetting agent)	0.9+0.3	6116	2.7
Simazine+paraquat (no wetting agent)	0.9+0.3	6337	1.9
Simazine+fluazifop-P (with wetting agent)	0.9+0.5	6561	2.4
LSD (5%)		NS	0.4

4.1.4.4 Effects of herbicides on nitrogen fixation by the clover

Differences in ^{15}N abundance between shoots and roots of the subterranean clover and barley grass were determined for each herbicide mixture, for each time and for each rate to determine the nitrogen fixation response (P fix) by clovers after spraying with different herbicides in comparison with the unsprayed control.

Type of herbicides: Analyses of variance of the results show that, in clover, the proportion of clover root N due to fixation (P fix) was significantly ($P < 0.01$) decreased by both herbicide treatments. In clover shoots, the P fix was also decreased by the herbicide application, but the difference was not significant, the lowest value (61%) occurred after early application of simazine-paraquat relative to the simazine+fluazifop-P treated and untreated plots. The decreased value of P fix N of the clover in the herbicide-treated plots may have resulted from the adverse effects of herbicide on nitrogen fixation by the clover despite the increased nodule count. Nodule number may have increased in an unsuccessful attempt by the plant to increase N fixation. This illustrates the unreliability of relying on nodule count to assess N fixation. Application of both herbicide mixtures resulted in different levels of total and fixed N in the subterranean clover shoots and roots in this experiment. The increases were greater after simazine+paraquat treatment than following simazine+fluazifop-P: however, the differences were not significant (Table 4.1.5).

Table 4.1.5 Effects of herbicide mixtures on the total N percent of barley grass and subterranean clover, and proportion of fixed N [P fix(%)] and total N fixed, of subterranean clover.

	Total N(%)				P fix(%)		Fixed N (kg/ha)	
	Grass		Clover		Clover		Clover	
Treatments	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.56	1.65	2.49	2.30	71	63	60	10
Simazine+paraquat	1.60	1.64	2.70	2.64	61	43	83	14
Simazine+fluazifop-P	1.63	1.90	2.37	2.14	63	46	70	9
L S D (5%)	NS	NS	0.20	NS	NS	6	NS	NS

***Rates of application*:** The percentage N in clover and grass and proportion of fixed N from atmospheric N_2 in clover roots when sprayed with simazine+paraquat at different rates showed considerable differences resulting from the different rates of application (Table 4.1.6). The P fix of clover was reduced following the low rate (0.7+0.25 kg a. i. /ha) and reduced still further at the high rate (0.9+0.3 kg a. i. /ha) of simazine+paraquat application: however, the differences in P fix between clover shoots sprayed at different rates were not significant. Application of herbicide significantly reduced only the proportion of the clover root N derived from fixation. The high rate was significantly different from the control at ($P<0.01$) whereas the low rate was significant at ($P<0.05$). The higher application rate of the simazine+paraquat mixture on the pasture significantly ($P<0.05$) increased total N and fixed N in clover roots.

Table 4.1.6 Effects of rate of application of simazine+paraquat mixture on the total N percent of barley grass and subterranean clover, and P fix(%) and total N fixed by subterranean clover.

Application	Total N(%)				P fix(%)		Fixed N (kg/ha)	
rates	Grass		Clover		Clover		Clover	
(kg a.i./ha)	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.56	1.65	2.49	2.30	71	63	60	10
0.75+0.25	1.65	1.80	2.74	2.32	67	50	83	12
0.9+0.25	1.60	1.64	2.70	2.64	61	43	83	14
L S D (5%)	NS	NS	NS	0.16	NS	9	NS	4

***Time of spraying*:** The earliest spraying (25/6/92) simazine+paraquat had the most effect on the N content of clover shoots and roots (Table 4.1.7). In comparison with the weedy control, simazine+paraquat treatment at rate of 0.9+0.3 resulted in a significant increase in total fixed nitrogen in clover shoots ($P< 0.05$), at all times of spraying except the last, and clover roots only following early spraying. These differences may be due to reduced competition from grass weed on the clover and not a direct effect of the application of herbicides.

The proportion (P fix) of clover shoot N was lower after the early application of herbicide (25/6/92) compared to later applications, of simazine+paraquat. Significant (P<0.05) differences occurred between early spraying (25/6/92) and later spraying on 24/8/92 as well as the unsprayed control. There was a progressive increase in P fix with later spraying dates with the exception of the 9/7/92 spraying. Simazine+paraquat application decreased the proportion of N from fixation in clover roots when compared to the unsprayed weedy control (again with the exception of the 9/7/92 treatment). The reduction was more marked on 25/6/92 than at any other time of herbicide spraying. Opposite results were obtained from the simazine+fluazifop-P mixture which decreased the clover P fix relative to the control following late spraying more than after early spraying. This decrease was significant (P<0.05) for the clover roots while not significant for the clover shoots relative to the control (Table 4.1.7).

Table 4.1.7 Effects of time of application of simazine herbicide combinations on the total N content (%) of barley grass and subterranean clover, and P fixed (%) and total N fixed, of subterranean clover.

Herbicides	Total N(%)				P fix(%)		Fixed N (kg/ha)	
and	Grass		Clover		Clover		Clover	
date of application	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.56	1.65	2.49	2.30	71	63	60	10
Simazine+paraquat								
25/6/92	1.60	1.64	2.70	2.64	61	43	83	14
9/7/92	1.63	1.66	2.43	2.51	69	68	79	12
24/7/92	1.53	1.99	2.66	2.46	65	46	75	9
6/8/92	1.64	1.68	2.64	2.35	68	54	71	9
24/8/92	1.66	1.68	2.55	2.30	70	56	66	8
Simazine+fluazifop-P								
25/6/92	1.64	1.90	2.37	2.14	63	46	70	9
9/7/92	1.62	1.79	2.46	2.44	63	30	63	4
L S D (5%) for:								
Simazine+paraquat	0.10	NS	0.13	0.17	9	6	9	4
Simazine+fluazifop-P	NS	NS	NS	NS	NS	6	NS	NS

Wetting agent: Addition of wetting agent to the herbicide mixture decreased clover shoot and root %N significantly (P<0.05) in simazine+paraquat treatments when compared to the same treatment without wetting agent. Significant decreases (P<0.05) in the %N of the grass shoots was obtained between simazine+paraquat without wetting agent and for all the herbicides tested with wetting agent. The addition of wetting agent to the herbicide mixtures decreased P fix in the clover shoots when compared to the same treatment without wetting agent and also when compared with the unsprayed control. The reduction was significant (P<0.01) for clover roots, but not significant for clover shoots. The yield of fixed N from the atmosphere increased in both shoots and roots of clover after herbicide treatment. This increase was greater when a wetting agent was added, but in neither case was the increase significant (Table 4.1.8).

Table 4.1.8 Effects of application of simazine+paraquat with and without wetting agent on the %N of barley grass, subterranean clover, and proportion fixed (%) and total N fixed subterranean clover.

	Total N (%)				P fix (%)		Fixed N (kg/ha)	
	Grass		Clover		Clover		Clover	
Treatments	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.56	1.65	2.49	2.30	71	63	60	10
Wetting agent (+)	1.60	1.64	2.70	2.64	61	43	83	14
Wetting agent (-)	1.41	1.66	2.52	2.46	69	62	73	12
L S D (5%)	0.18	NS	0.16	0.21	NS	19	NS	NS

The overall effects of the herbicide combinations showed that herbicide treatment led to increase in accumulation of fixed N in clover shoots but not in clover roots, the percentage of plant total nitrogen in clover roots was slightly lower than that of shoots for all the treatments, where the percentage of plant nitrogen in grass roots was higher than that of shoots in all cases (Table 4.1.9). The $\delta^{15}N$ of clover roots was consistently higher than that of the shoot nitrogen in the plots (P fix in clover roots was lower than in clover shoots). The ^{15}N content of the soil N is enriched relative to air, so a low ^{15}N content in the clover results from a high level of fixed N in the clover. Thus there is a higher accumulation of fixed N in clover shoots than in clover roots.

Table 4.1.9 The overall effects of the herbicides on the nitrogen accumulation by barley grass and the subterranean clover.

Treatments	Total N(%)				Pfix(%)		Fixed N (kg/ha)	
	Grass		Clover		Clover		Clover	
	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Control	1.56	1.65	2.49	2.30	71	63	60	10
Simazine+paraquat	1.58	1.72	2.59	2.42	68	55	74	11
Simazine+fluazifop-P	1.63	1.85	2.42	2.29	63	38	66	7

4.1.4.5 ^{15}N abundance of soil, legume and reference plants at the experiment site

The measurement of ^{15}N, total N percent and inorganic nitrogen in soil were based on the analysis of the 88 cores. The results from $\delta^{15}N$ analysis shows that the $\delta^{15}N$ of total N increased with depth, however the total percentage N and inorganic nitrogen tended to decrease with depth.

The minimum $\delta^{15}N$ value obtained for the soil total N across the area was over 6 ‰, and the total mean value of the profile sampled was 6.52 ‰. Recorded values for the mineral N fraction ranged from 40 to 183 µg N/g soil, and with a total mean value of 86 µg N/g soil, lower than that for the total soil N mean value of 0.09 % (Table 4.1.10).

Table 4.1.10 Mean values of total N (%) and available soil N(µg N/g soil) and ^{15}N (‰) of the experimental area at Kapunda in 1992.

Soil depth(cm)	Total N (%)	Available N (µg N/g soil)	^{15}N (‰)
0-5	0.17	134	6.14
5-10	0.08	114	6.33
10-20	0.05	56	6.69
20-30	0.04	41	7.46

The ^{15}N of the plant nitrogen of sub clover and reference plant are compared with the soil ^{15}N and the data are summarised in Table 4.1.11. Values for both soil and plant varied appreciably between most plots in the experimental area.

Table 4.1.11 Total N, natural ^{15}N abundance of soil total N and plant total N from the control (unsprayed) plots (mean values).

Subterranean clover		Barley grass		Soil	
Total N (%)	^{15}N (‰)	Total N (%)	^{15}N (‰)	Total N (%)	^{15}N (‰)
2.55	2.53	1.61	4.46	0.09	6.85

Variability of $\delta^{15}N$ and percent N in reference plants across the plots: Total N% and ^{15}N (‰) of the barley grass shoots collected across the treated plots during the data collection, were measured to estimate the variability of ^{15}N (‰) and to study the sensitivity of the reference plant to variation of soil N around the experimental area. A slight variation was obtained between the plots in respect of (‰)^{15}N from 3.60 to 4.17. Such a variation should not affect measurements of P fix provided paired legume and reference plants were collected and used to calculate P fix, although % N of the barley grass shoots varied markedly between 0.67 and 1.94 (Table 4.1.12).

Table 4.1.12 Total N% and ^{15}N(‰) of barley grass shoots from six evenly-spaced positions (a,b,c,d,e,f) on the control plots (sampled from North to South).

	a		b		c		d		e		f	
Plot No	Total N%	$\delta^{15}N$	Total N%	$\delta^{15}N$	Total N%	$\delta^{15}N$	Total N%	$\delta^{15}N$	Total N%	$\delta^{15}N$	Total N%	$\delta^{15}N$
1	1.19	3.92	1.33	3.76	1.02	3.65	0.67	3.72	1.04	4.14	1.32	3.86
14	1.21	3.89	1.12	3.84	1.62	4.14	0.97	3.68	0.92	4.02	1.10	3.73
22	0.87	4.07	1.03	4.17	1.12	4.16	1.01	4.03	1.94	4.03	1.41	4.02
33	1.32	3.91	1.22	4.13	1.04	3.66	0.98	4.15	1.17	4.04	1.36	3.74
37	1.33	4.03	1.37	4.06	1.25	4.06	1.14	4.17	1.44	3.60	1.50	4.05

The N% and $\delta^{15}N$ of the barley grass along with other non-fixing plants in the experimental area were measured to determine the variation of ^{15}N between non-fixing plants in the area. The plants were at different stages of maturation at the time of sampling. For example, grasses had matured whereas the wireweed and capeweed were still green. All of the grass species had a higher $\delta^{15}N$ level than the broad leaf plants. The variable $\delta^{15}N$ content of the different positions across the plots (Table 4.1.12) and also the variation between non-fixing plants (Table 4.1.13) indicated the necessity of pairing N_2-fixing plants with reference plants when estimating N_2 fixation by the ^{15}N dilution method.

Table 4.1.13 The ^{15}N of grass and broad leaved weed tops sampled on 25/9/92 on the experiment site, Kapunda.

Plants	$\delta^{15}N$(‰)
Barley grass (*Hordeum sp.*)	3.6
Brome grass (*Bromus sp.*)	3.7
Silver grass (*Vulpia sp.*)	3.1
Ryegrass (*Lolium rigidum*)	4.5
Wild oats (*Avena fatua*)	3.9
Salvation Jane (*Echium plantagineum*))	2.5
Geranium (*Erodium botrys*)	2.5
Wireweed (*Polygonum aviculare*)	2.0
Capeweed (*Arctotheca calendula*)	2.9

4.1.5 DISCUSSION

The results of this experiment show that the type of herbicide, its rate and time of application have considerable impact on the production of pasture and amount of nitrogen fixed by the legume component.

The herbicide mixture simazine+paraquat and simazine+fluazifop-P mixture at different rates and times of application significantly controlled associated grasses, reducing grass DM yields up to 76.6 %, and increased dry matter yields of the subterranean clover up to 46% compared to the unsprayed control treatments. The increased subterranean clover DM yield was probably caused by reduced competition from the grass component in the treated plots. Ledgard and Steele (1992) indicated that in a grass/legume pasture, any factor that retards the grass component, can enhance the competitiveness of the legume and further reduce the yields of the associated grasses.

Simazine+fluazifop-P was more efficient in controlling grasses than was the simazine+paraquat treatment. This was expected as the selective nature of fluazifop-P causes death of the grass component (Thorn and Perry 1983). The change in the grass and subterranean clover contents of the pasture observed between the different herbicide combinations used in this experiment is comparable with those observed by Leys and Plater (1993). They reported that when controlling annual grass weeds (mainly barley grass) in subterranean clover pasture, simazine alone tended to slightly reduce grass densities, but the addition of paraquat to simazine significantly ($P<0.05$) reduced the grass densities compared with the unsprayed treatment. Furthermore, the addition of fluazifop-P to simazine also significantly ($P<0.05$) reduced grass densities compared to simazine alone. In our experiment, root densities of grass were not measured, but reductions in grass root dry weights of up to 87 percent were recorded. There was a marginal reduction of sub clover root densities in the herbicide-treated plots.

In this Kapunda experiment, spraying the herbicide mixtures at different times altered the proportions of clover and grass components in the sward by differing amounts. This suggests that the stage of plant growth at which herbicides were applied, affected the plant response to the herbicide. Application of simazine+paraquat late in June, when the sward was at the early stage of growth, significantly ($P<0.01$) reduced the grass component and caused increases in subterranean clover yields, but if sprayed late in August when plants were at a later stage of the growth, the herbicide was less effective on grasses, in part because of lower percentage contact with grasses. Also older grasses are more tolerant of paraquat. The reduced grass control benefited the clover less. Others have also reported that in a pasture containing subterranean clover and grasses, the application of paraquat in June gave a higher clover content than when applied in August (Barrett *et al.* 1973). The lower impact of late applications of herbicides on grass weeds is probably because most grasses were more advanced in growth and therefore were more tolerant of the herbicides (Sagar *et al.* 1982). There is also a lower percentage of contact between grass and herbicide. It has been suggested that clover recovers from herbicide treatment with time (Leys and Plater 1993). This also may explain the higher production of clover with early application of herbicide. However the major factor leading to the imposed growth of sub clover is likely to be the reduced competition for grass following control by herbicides.

The results show that the early (25/6/92) application of the simazine+paraquat with the low rate (0.75+0.25) was effective and reduced the grass yield by up to 528 kg/ha DM relative to that of late spraying (24/8/92). The high rate (0.9+0.3) of simazine+paraquat application reduced the grass dry matter up to 1032 kg/ha. These results indicate that the early spraying of the herbicide allowed the use of lower doses of the herbicide, but a much better result was obtained with the full rate of herbicide applied early.

Herbicide application without wetting agent resulted in higher grass and lower subterranean clover dry matter yields. Various additives (1 L/ha or 3 L/ha EK-VEG, a 90% vegetable oil, or 3 L/ha Sun-oil 11E, a 98.8% mineral oil) to the herbicide alloxydim-sodium at four doses (from 0.007 to 1.8 kg a.i./ha) also produced a significant enhanced activity on barley grass (*Hordeum spp.*) (Kudsk *et al.* 1987). Predictably, in this Kapunda experiment, the addition of wetting agent to herbicide, brought about better control of grass. This may elevate the phytotoxicity of the herbicide by, for example, increasing the spreading and retention of the herbicide on the plant (Johnstone 1973; Hull *et al.* 1982).

Total nodule number in the clover component of the pasture was increased in plants treated with herbicide and there were quantitative differences in N_2 fixation. These results support those of Pate and Dart (1961) who suggested that the N-fixing efficiency of nodules can be altered without any associated change in nodule numbers. The increase in nodule number in subterranean clover in the treated plants was presumably because of the reduction in competition from the grass. The variability in nodule score between treated and untreated sub clover plants and lack of correlation between nodule number and proportion of fixed N suggest that nodule score is not a reliable indicator of the nitrogen-fixing ability of the clover. The difficulties in extracting roots with nodules intact from the soil, may also be a cause for the lack of a correlation.

In this Kapunda study, total N% and ^{15}N abundance values of legume shoots were different to those of the corresponding legume roots. This variation of ^{15}N abundance in plant parts has also been reported by other authors (Steele *et al.* 1983; Yoneyama *et al.* 1986 and Ledgard 1989).

The amount of nitrogen fixed in subterranean clover for the herbicide-treated plots was consistently higher than that in the untreated plots. The higher amount of fixed nitrogen in the subterranean clover after herbicide application (Tables 4.1.4, 4.1.6) is associated with greater clover DM in the treated plots, and results from reduced competition from associated grasses. The results obtained from the time of spraying experiment on the proportion of fixed nitrogen by clover show that where clover growth was increased, through a decrease in yield of the associated grasses produced by the herbicide treatments, the proportion of fixed nitrogen was decreased (in contrast to the amount of N fixed) (Figure 4.1.5). The decreased proportion of grass may have left more inorganic N in the soil, thus allowing the clover to get a higher proportion of its nitrogen from the soil, decreasing the proportion of nitrogen fixed. Alternatively, the non-selective herbicide may have had a harmful effect on the fixation process thus reducing the proportion of fixed nitrogen and the effect of the herbicide was highest when applied early.

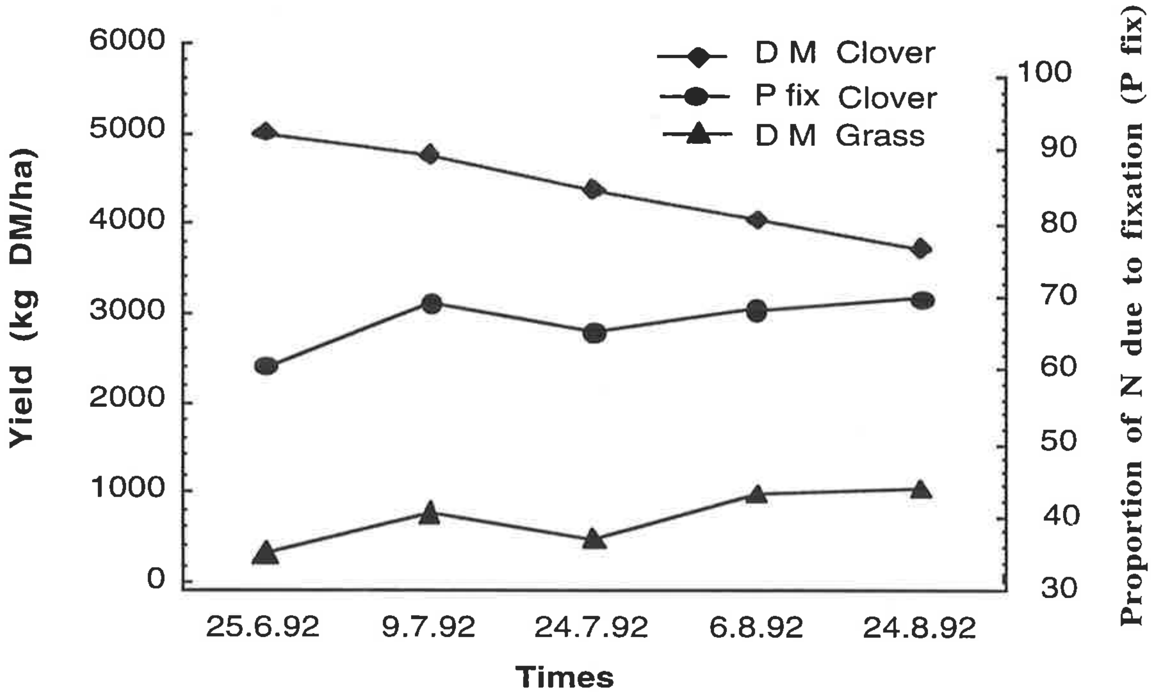


Figure 4.1.5 Effects of simazine+paraquat sprayed at different times on dry matter yield of grass and clover shoots and proportion (%) of N due to fixation in clover.

In this experiment it was not possible to determine the responses of subterranean clover to the herbicides alone because of the complicating factor of reduced competition from grasses causing an increase in the dry matter yield of subterranean clover. These herbicides have been previously reported to damage caused from herbicide phytotoxicity on subterranean clover in pastures. Leys *et al.* (1991) using a subterranean clover pasture more than 10 years old, containing high densities of *Vulpia* spp. and low densities of catsear spp., reported a moderate yellowing and reduction in dry matter of subterranean clover by simazine applied late in May and July at the rate of 1.0 kg/ha. Leys and Plater (1993) using visual assessments of grassy pastures, with good stands of subterranean clover, observed damage to sub clover 4 weeks after application of paraquat alone (400 g a.i./ha), and mixture of simazine plus fluazifop-P (500+20 g a.i./ha) or a estimated a 37-75% reduction in herbage production in a clover/ grass pasture due to herbicide. Cuthbertson (1964) also observed severe adverse effects of these chemicals on subterranean clover. Leys and Plater (1993) also found severe leaf burn and retarded growth of a weed-free sward of subterranean clover and a significant ($P<0.05$) reduction of dry matter, in 12 of the 21 herbicide treatments.

In addition to the competitive effects of the grass component of the pasture on the clover, soil conditions may also have affected growth and nitrogen fixation in the pasture. Reports from a number of investigators indicate that variations in levels of soil inorganic nitrogen in a legume pasture may influence nodulation and nitrogen fixation (Butler and Ladd 1985a). These effects depend on the amount of N, species of legume, strain of rhizobium and factors affecting plant growth: small doses may have either positive or negative effects. Harper and Gibson (1984) suggested that growth of the legume and its nitrogen fixation is often increased by small doses of N, especially if the added N is available during the time between exhaustion of seed reserves and establishment of the root system. Ledgard and Steel (1992) reported that at a similar stage of grass/legume growth in a pasture, the grass takes up more soil nitrogen reducing the inhibitory effect of soil nitrogen on biological nitrogen fixation. Butler and Ladd (1985a) indicated that nitrogen fixation is affected by the competitive effects of grass on legume through decreased soil N levels, in a grass/legume pasture with a high level of soil N. A decrease in proportion of the associated grasses, produced by the herbicide, may increase the availability of soil N leading to increased uptake of soil available N, reducing the need for the legume to fix nitrogen. Butler and Ladd (1985b) found no change in nitrogen fixation of a medic when grown with decreasing numbers of ryegrass plants when grown at high levels of soil N compared with low levels of soil N. In contrast, in a grass/legume pasture with a low level of soil nitrogen, increasing the proportion of grasses may cause a decrease in the amount of nitrogen fixed by the legume to a greater extent than that expected from the decrease in legume production (Butler 1988). In the Kapunda experiment, the available soil mineral N at the surface was between 85 and 183 μg N/g soil. Comparison of these results with those of other workers is difficult because of the variety of ways in which the levels of mineral N are reported and the large number of factors affecting the response to combined N. Gibson and Nutman (1960) and Dart and Wildon (1970) reported that soil N concentrations of up to 100 ppm in the solution may have either a stimulating, neutral or inhibiting effect on nodulation, but concentrations much in excess of this are usually inhibitory. However, the degree of nitrate inhibition of the nitrogen fixation process of legume plants by combined N varied for different legume-*Rhizobium* combinations and depended on the amount of the combined N, and the time of utilisation (Gibson 1971; Munns 1977; Evans 1982). In the field, there are other factors affecting the symbiotic response to nitrate in soil solution, for example soil moisture (Streeter 1972) and water stress (DeJong and Phillips 1982). Therefore, it is difficult to assess the degree of the inhibitory effect of soil N in this Kapunda experiment.

Barley grass was chosen as a reference plant from the range of non-fixing plants tested since it was widespread across the plots and consequently allowed the best opportunity for pairing reference and N-fixing plants that were growing close together. Barley grass also removed N with an intermediate $\delta^{15}N$ content compared to other species (Table 4.1.13). The $\delta^{15}N$ content of the grasses (Table 4.1.13) may reflect the earlier maturity date of these relative to the broad leaf plants, or may reflect some inherent discrimination difference shown by monocot and dicot species. An understanding of the reason for this difference could assist in the selection of the most suitable reference plants.

Soil analyses show that total N, ^{15}N abundance of total N and inorganic soil N varied over the site and with depth. Total N varied from 0.04 to 0.17 % and, as expected, was higher in the 0-5 cm than in the 20-30 cm. depth. It has been reported that natural ^{15}N abundance of total N in soil is generally less than 15 ‰ and usually 5-12 ‰. Also soil available N in the experimental area varied from 85 to 183 µg N/g soil and the amount decreased with depth, while the natural abundance of $\delta^{15}N$ of total soil N was higher at 20-30 cm than the surface 0-5 cm (7.46 and 6.14 ‰ respectivcly). Wilson (1981) and Ledgard *et al* (1984) have also found increases in δ^{15} N content of total soil N with depth under pasture. Shearer and Kohl (1986) suggest drainage and litter are responsible for these differences. Higher $\delta^{15}N$ levels in soil N may be found in lower slopes, because water logging causes more denitrification on the lower slopes than on upper slopes, leading to increased losses of the lighter ^{14}N relative to ^{15}N. Also surface soil under brush often has a lower ^{15}N abundance than soils in open areas, which according to Shearer and Kohl (1986) may result from litter deposition. This could also result from lower losses of N from brush areas and thus less loss of ^{14}N relative to ^{15}N.

4.1.6 CONCLUSIONS

Several studies have shown interactions between herbicide application legume production and nitrogen fixation. In the Kapunda study, herbicides reduced the grass weeds and reduced the suppression of clover by the grass, thus improving the growth of the clover and increasing N fixation. It is not possible using the available data to determine which is the dominant mechanism affecting the N_2-fixing process.

The results indicate that stimulatory or inhibitory effects of the herbicides on growth and N_2 fixation are dependent on herbicide type and concentration, the use of a wetting agent and the time of herbicide application. Early spraying of herbicide on the pasture allowed the use of lower doses of herbicide and invariably led to the best yield from the legume. The results support the hypothesis that efficacy of herbicides is altered by factors such as rate of application, time of spraying and addition of wetting agents, and that more-careful use of herbicides would allow more efficient exploitation of biologically-fixed N for the maintenance of soil fertility.

A study of the side-effects of herbicides on biological nitrogen fixation is important in ensuring the safe and efficient use of herbicides in legume pastures. Such knowledge will improve biological nitrogen fixation, and may directly reduce dependence expensive N fertiliser inputs on farms. Indirectly, this knowledge may reduce dependence of herbicide.

There is a need for sustainable farming to maintain soil fertility using renewable natural resources particularly in developing countries where N fertilisers are not readily available or affordable. There is an ongoing need to continue efforts to improve the efficiency of herbicide use, as well as to search for more-acceptable and effective biological, mechanical and chemical methods of weed control.

4.2 EXPERIMENT 4. ISOTOPIC FRACTIONATION DURING N_2 FIXATION BY *MEDICAGO TRUNCATULA* (cv. PARAGGIO) AND *TRIFOLIUM SUBTERRANEUM* (cvv. MOUNT BARKER AND CLARE). (Location: Glass house at Waite Institute)

4.2.1 INTRODUCTION

While it is commonly assumed that the fixed nitrogen associated with symbiotic N_2 fixation has the same isotopic composition ($^{15}N/^{14}N$) as atmospheric N_2 (Junk and Svec 1958) and the isotopic composition of atmospheric N_2 is found to be essentially constant at 0‰ (0.3663 atoms %^{15}N) throughout the world (Mariotti 1983), infact the isotopic composition of N in nitrogen-fixing legumes is affected by isotopic discrimination during N_2 fixation, and ^{15}N abundance of legume nitrogen derived from the atmosphere is altered by this

B is defined as the ^{15}N concentration of the legume when grown with atmospheric N_2 as the sole source of nitrogen.

Bergersen and Turner (1983) reported that the use of natural abundance methods to estimate biological nitrogen fixation requires the value of the *B* factor to calculate the proportion of plant nitrogen derived from N_2 fixation (P fix) and that it is important to establish the level of isotopic fractionation during N_2 fixation by legumes. Unkovich *et al.* (1994) have concluded that for progressive measurements of fixation throughout the growing season, the ^{15}N values of fixed N and reference plants at each specific growth rate need to be matched against the *B* value for that stage of plant growth.

This experiment described here was undertaken to obtain the *B* values for isotopic fractionation during nitrogen fixation for the three major pasture legume cultivars used in the research for this thesis. Thus, nitrogen fixation estimates, corrected for isotope discrimination, could be calculated for all field experiments described in this thesis.

4.2.2 MATERIALS AND METHODS

Seeds of the study species *Medicago truncatula* (cv. Paraggio) and *Trifolium subterraneum* (cvv. Mount Barker and Clare) were surface sterilised (rinsed with 95% ethanol for 10 seconds, soaked for 3 minutes in 0.2% $HgCl_2$ and rinsed thoroughly in distilled water). The seeds were pre-germinated in distilled water at 20°C for medic and 25°C for clovers for one day. Each seedling was inoculated with peat inoculum mixed with deionised water as a slurry; the same strains of *Rhizobium* were used as in the field experiment, viz. a culture of *Rhizobium meliloti* WSM540 Group A for medic and *Rhizobium trifolii* WU95 Group C for subterranean clovers (Inoculant Service, Victoria, Australia). Twenty seedlings of each species were transplanted at the normal emergence stage into free-draining sand in square (15 ×15 cm) black plastic pots on 23 April 1994. The pots had been sterilised with 95% ethanol, dried and filled with about 3 kg of river sand which had previously been washed and steam sterilised. The sand surface was covered with white polythene beads to reduce water loss and prevent algal growth. Twenty five replicates of each of the three species were used. The plants were grown in a naturally-lighted glasshouse where maximum temperature was controlled at 26°C and minimum at 20°C. The pots were watered to field capacity daily for one week with deionised water. Thereafter, nitrogen-free nutrient solution was supplied each morning and in the afternoon they were watered with distilled water. The sand in the pots drained rapidly to field capacity (from a water holding capacity of 10%). Nutrient solution was prepared from stock solution with deionised water and $\frac{1}{2}$ strength Hoagland's solutions (0 mM for nitrate) containing(mg/L): $CaSO_4.2H_2O$ (430); KH_2PO_4 (33.75); K_2SO_4 (217.8); $MgSO_4.7H_2O$ (245); $FeSO_4.7H_2O$ (39.8); EDTA (47.64); $MnCl_2.4H_20$ (1.81); $ZnSO_4.7H_2O$ (0.22); $CuSO_4.5H_2O$ (0.08); $Na_2Mo\ O_4.\ 2H_2O$ (0.12); $H_3\ BO_3$ (2.86). The pH of the nutrient was adjusted to 7.0 using Ca $(OH)_2$.

4.2.3 DATA COLLECTION

Five harvests were taken from each of the cultivars at 15, 30, 45, 60, 75 days after transplanting to determine the trend of the ^{15}N level in the legume N with time and hence the *B* values. Harvests involved removal of the plants from the sand by washing with water and separation of plants into shoots and roots. Dry weights were determined, and finely-ground plant materials were analysed for total N and ^{15}N as described for the previous experiment.

4.2.4 RESULTS

The data in Figure 4.2.1 show the pattern of nitrogen accumulation of the subterranean clovers and barrel medic during the experiment. The difference in the plant N between the clover species and the medic was significant ($P< 0.05$) during growth and the differences tended to increase with time.

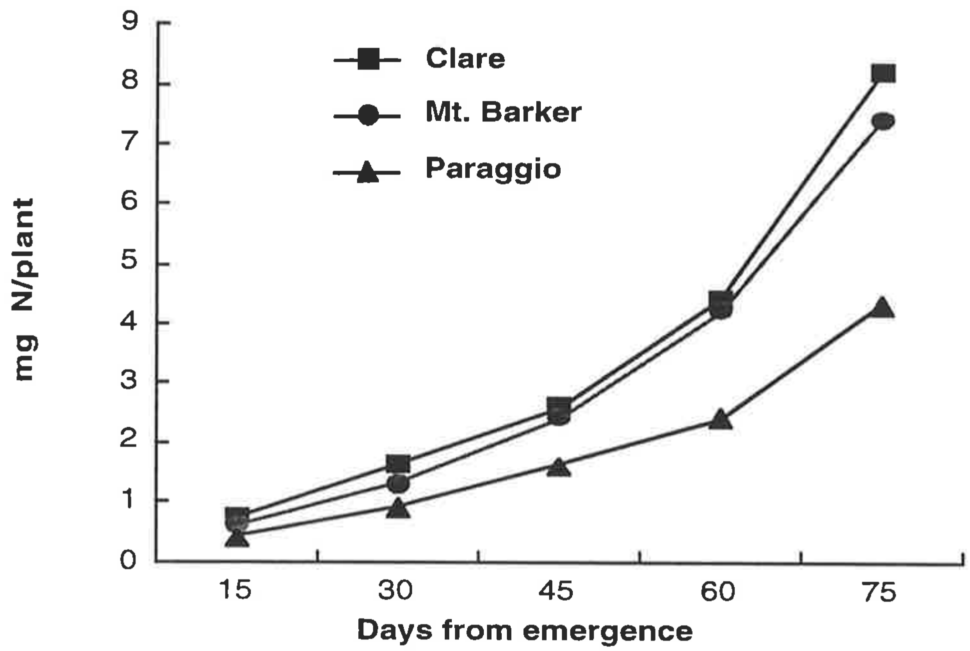
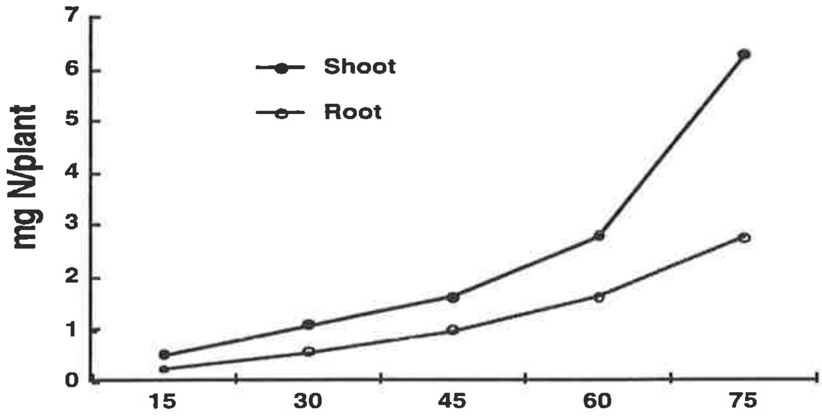


Figure 4.2.1 **The time course of accumulation of nitrogen by *Trifolium subterraneum* (cvv. Mount Barker and Clare) and *Medicago truncatula* (cv. Paraggio) completely dependent on symbiotically fixed-N_2.**

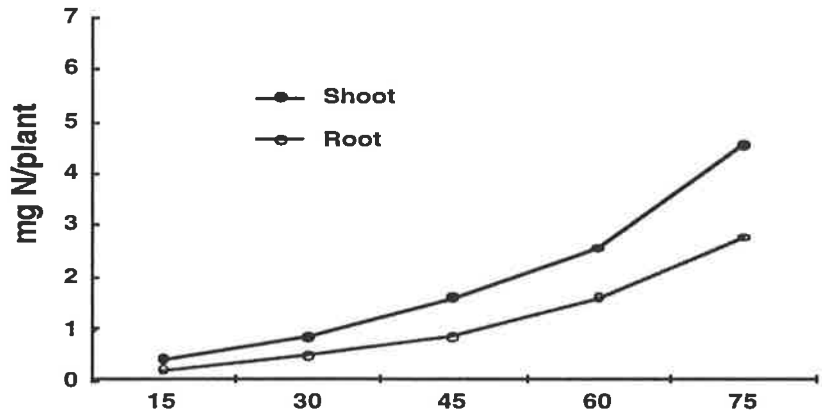
Root materials contained a lower percentage of plant nitrogen than shoots in each of the legumes (Figure 4.2.2).

The abundance of ^{15}N in shoot and root biomass showed a sharp decrease from the first harvest at 15 days, and apparently reached an equilibrium 60 days after sowing (Figure 4.2.3).

(a) Clare



(b) Mt. Barker



(c) Paraggio

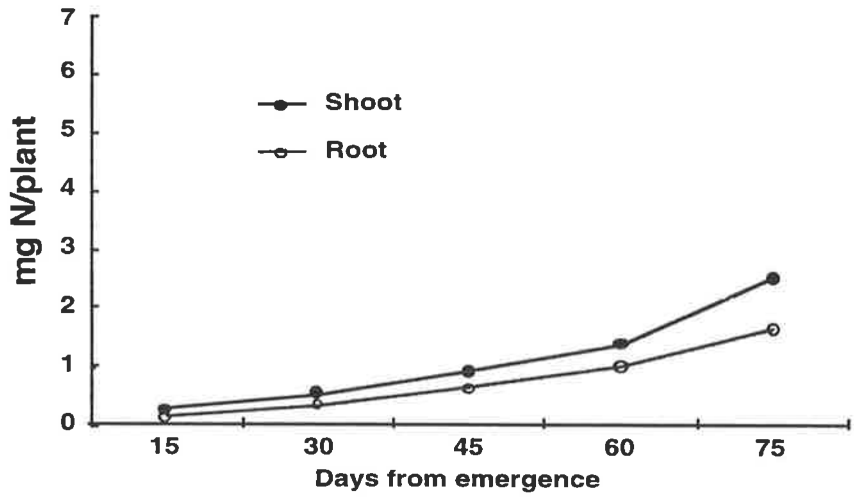
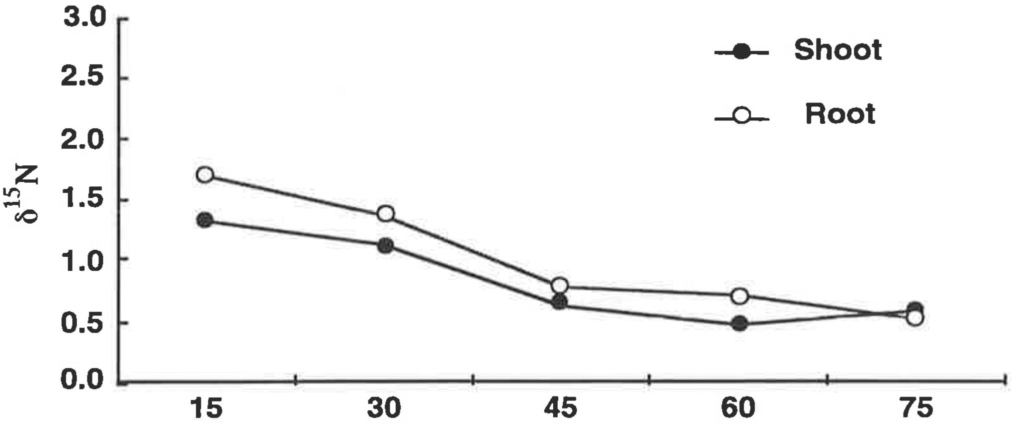
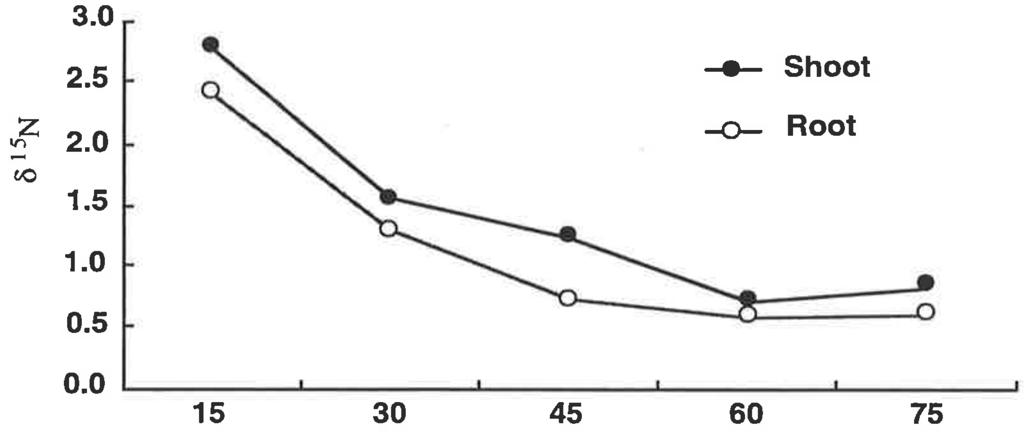


Figure 4.2.2 **Partitioning of nitrogen accumulated during growth between shoots and roots for subterranean clover cv. Clare and Mt-Barker and barrel medic cv. Paraggio.**

(a) Clare



(b) Mt. Barker



(c) Paraggio

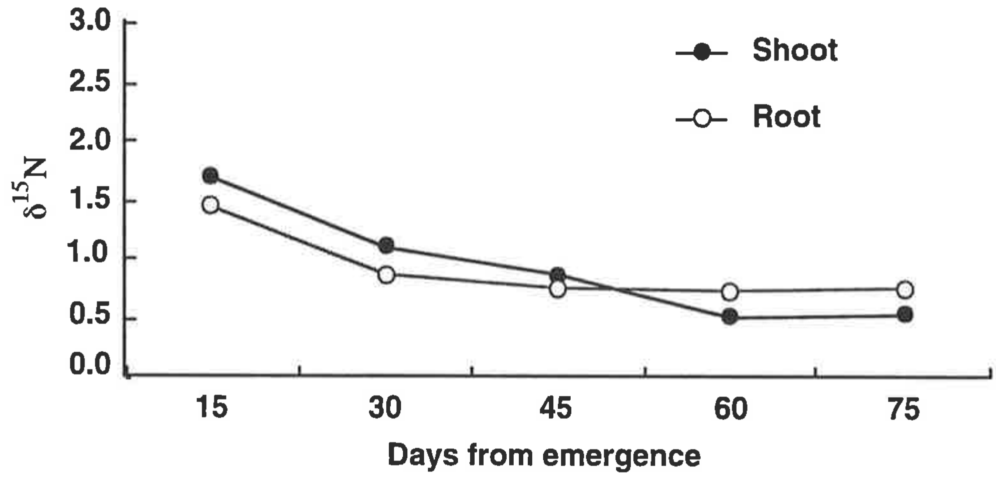


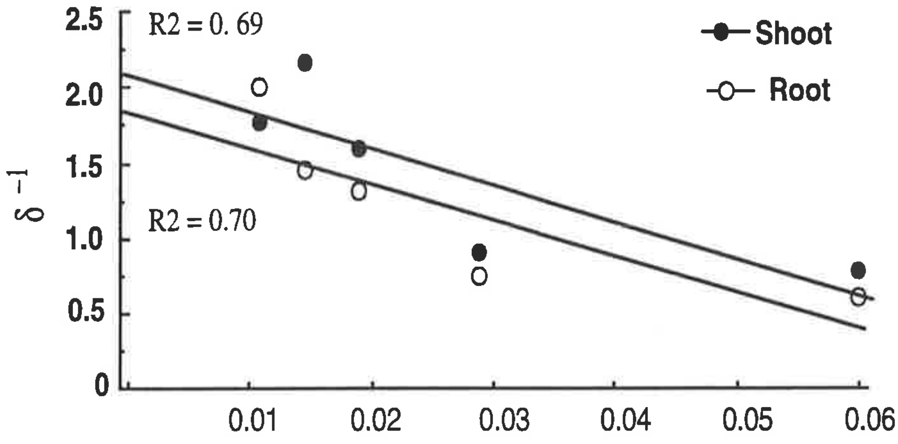
Figure 4.2.3 The time course of decline in $\delta^{15}N$ of the total nitrogen in shoot and root of entire symbiotically-dependent plants.

However, when the $\delta^{15}N$ in the nitrogen accumulating with time in each plant part were compared, significant differences were found between shoots and roots of the three legumes at all harvests (except in roots at Harvest 2)

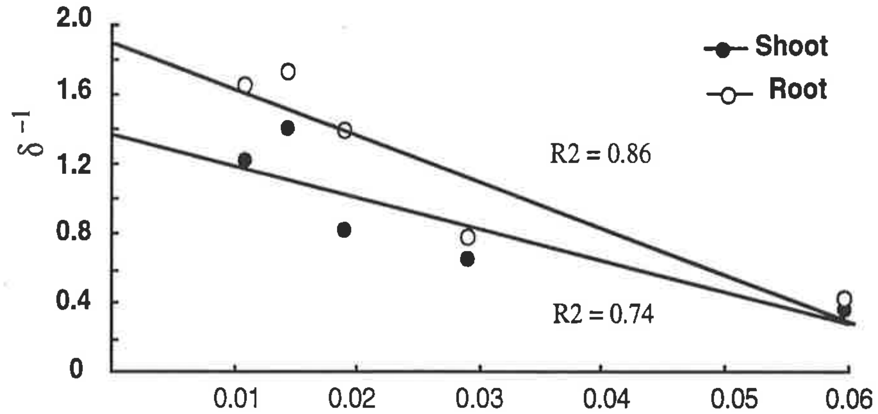
4.2.4.1 Calculation of *B*

When the seedlings were grown in a nitrogen-free nutrient solution in the glass house, initially the N in the seedlings came from the seeds, then between germination and nodulation from seed and from any N in the medium. This caused an initial high level of $\delta^{15}N$ in the plant, which subsequently declined sharply with time once the major source of N came from atmospheric nitrogen fixation (Figure 4.2.3). To calculate any isotopic discrimination associated with the fixation process, the isotopic fractionation factor (B) associated with N_2 fixation by the pasture legumes used in this experiment was obtained from the intercept of the regression lines between $1/\delta^{15}N$ and 1/time from day 15 onwards (Bergersen and Turner 1983) (Figure 4.2.3). This method graphically extrapolates the data to estimate the ^{15}N level at infinite time when all seed and medium N is exhausted (calculated from the intercept on the Y axis). The values for B calculated for shoots and roots of each species were lower than values for $\delta^{15}N$ obtained at days 60 and 75 (after adjustment for seed nitrogen) and are respectively 0.48, 0.56 for the Clare shoots and roots, 0.53, 0.72 for Mt. Barker shoots and roots and 0.50, 0.62 for Paraggio shoots and roots.

(a) Clare



(b) Mt. Barker



(c) Paraggio

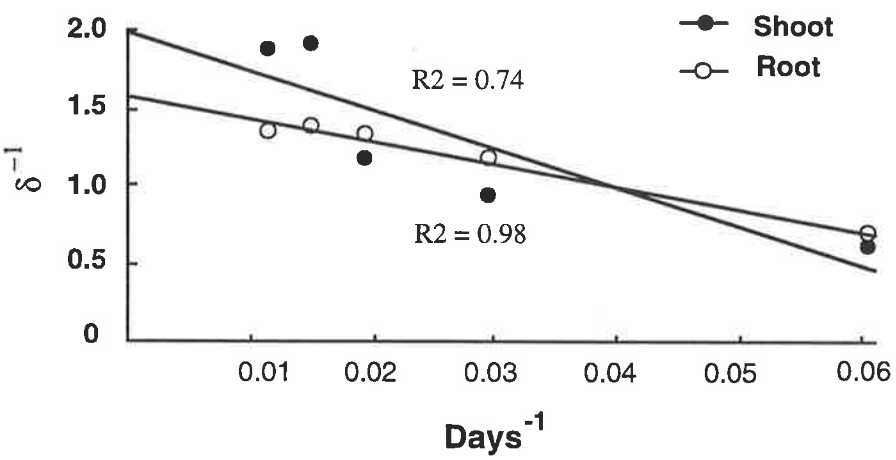


Figure 4.2.4 **Regression of δ^{-1} vs. time^{-1} for estimation of the equilibrium value of δ for N_2 fixation.**

4.2.5 DISCUSSION

The time course of accumulation of nitrogen in the clovers and medic showed that there were small but significant differences in total nitrogen between the clovers and the medic during plant growth up to 75 days after sowing (Figure 4.2.1). The differences in the ability of the clovers and medic to fix nitrogen are a feature of each plant, which may be associated with differences in dry matter production, or nitrogen content of the soil. The nitrogen accumulation also differed between shoots and roots in each species (Figure 4.2.2).

In this experiment, shoots and roots were prepared separately for estimation of the isotopic fractionation during N_2 fixation, as many authors, including Steele *et al.* (1983), Yoneyama *et al.* (1986) and Ledgard (1989) have agreed that natural ^{15}N abundance in plant tops varied compared to that in plant roots depending on the host plant features and/or *Rhizobium* strain. Since the *B* value was used for the calculation of the proportion of fixed nitrogen in the ^{15}N natural abundance method, estimation of N fixation using the shoot *B* value only may be inaccurate.

The $\delta^{15}N$ recorded was higher at the first harvest at 15 days after sowing and then decreased to a relatively constant value at 60 and 75 days in both shoots and roots of the three cultivars (Figure 4.2.3). The initial higher $\delta^{15}N$ level would not be due to N_2 fixation, as nodules had not been formed at that stage of growth. This probably was due to the nitrogen initially present in the seed, or by traces of nitrogen present in putatively nitrogen-free growth media. The constant level of ^{15}N late in the growth should reflect the isotopic discrimination during the N_2 fixation process (Bergersen *et al.* 1988).

The isotopic fractionation factor (*B*) was found to be different for the different legume cultivars. This may depend on the different strains of rhizobium used, or on difference in the $\delta^{15}N$ content of the seed, or be due to a characteristic of the host plant. However, this variation between cultivars is in general agreement with studies by various authors (e.g. Bergersen *et al.* 1988; Shearer and Kohl 1989). In subterranean clover (Mt-Barker), the *B* value of 0.72 obtained here for shoots was greater than that (-0.9) for shoots reported by Unkovich *et al.* (1994). In contrast, the *B* value (0.53) recorded in roots was lower than the value in root material (0.9) reported by the same authors. The values of *B* obtained for subterranean clovers Clare and Mt-Barker are lower than those reported by Bergersen and Turner (1983). A value of *B* greater than 1 appears in the literature (e.g. Amarger *et al.* (1979).

Various authors (Amarger *et al.* 1977, 1979; Mariotti *et al.* 1980; Kohl and Shearer 1980; Yoneyama *et al.* 1991 and Unkovich *et al.* 1994) have shown different *B* values for different cultivars and even for the same plant cultivars. These differences between authors may be due to differences in rhizobial strains, in isotopic fractionation during sample preparation or due to differences in methods of estimating $\delta^{15}N$. Therefore, in the natural ^{15}N abundance methods for calculating the proportion of fixed nitrogen, estimation of the *B* value under controlled conditions. The *B* value obtained from the reciprocals of $\delta^{15}N$ and time (Figure 4.2.4) in this experiment were used to adjust the measured value of the proportion of fixed N to estimate the actual value for the proportion of nitrogen due to N in fixation by the pasture legumes in the experiments of this study.

CHAPTER 5

5.1 EXPERIMENT 5. THE EFFECT OF MECHANICAL DEFOLIATION AND HERBICIDE 2,4-DB APPLICATION ON N_2 FIXATION, HERBAGE YIELD AND SEED PRODUCTION OF PARAGGIO BARREL MEDIC AT THREE SOWING DENSITIES.

(Location: Field at Waite Institute).

5.1.1 INTRODUCTION

On the neutral to alkaline soils of southern Australia, and in some other countries with Mediterranean-type climate, medics are used in the ley farming system for improving greenfeed production and maintaining soil N (Carter 1975,1989; Crawford *et al.* 1989). Benefits from this system of farming can be maximised through an understanding of the variation in the rate of N_2-fixation by medics during the growing season.

Weed control using herbicides is an important factor influencing plant yield and symbiotic N_2 fixation in pastures. Herbicide use can reduce the weed component in a pasture, and may lead to increased herbage yields and N_2-fixation. It may also have detrimental effects on biological nitrogen fixation by pasture legumes. In previous experiments, the impact of herbicides on nodulation, nitrogen fixation and plant yield of pasture legumes was studied. The results showed that, in the grass/legume pasture, reduction in the grass component of the pasture resulted in an increased dry matter yield of legume plants. However, in legume pastures, the herbicide generally caused a reduction in legume yield and N_2-fixation. The results also showed that different species of pasture legumes were affected to differing degrees by different herbicides, different concentrations of the same herbicide and the growth stage at which the herbicide was sprayed.

Mechanical defoliation is also a practical and sustainable method of weed control in pastures, The rationale behind this method, which involves the cutting of both weed and legume, is that the success of each component will depend on its ability to survive and recover from defoliation. For example, annual grass weeds, which are major weeds of legume pastures in South Australia, produce flowering tillers throughout the growing season: if these tillers are removed by cutting them were low, their survival will depend on their ability to produce new basal tillers.

Defoliation has been reported to induce a rapid decline in N_2 fixation by legumes (Davidson *et al.* 1990; Gordon and Kessler 1990). Responses of N_2 fixation to defoliation in legumes is also influenced by environmental conditions, frequency and time of defoliation and differs between species or cultivars (Motazedian 1984). Fisher (1973) studied the effects of defoliation at different times and heights of cutting on the growth and development of the annual pasture legume, Townsville stylo (*Stylosanthes humilis*), in irrigated swards and reported that frequent defoliation increased relative growth rate compared with the control and less-frequently defoliated treatments. He suggested that sustained grazing during the growing season may be important in controlling grass weeds sensitive to grazing, without greatly reducing pod yield or total dry matter production of Townsville stylo. Butler (1986) found that repeated defoliation markedly reduced N_2 fixation by medic plants and in many situations a linear relationship between medic yields and amount of fixed N was found. Cutting management has also been shown to influence yield of herbage (Spedding 1971). Brougham (1956) found that the amount of herbage including leaf material before and after defoliation influenced the rate of regrowth following defoliation. In general, leaf material present prior to defoliation and the foliage remaining after defoliation influence regrowth following defoliation.

Plant density is a major consideration in the evaluation of annual legumes for pasture production, N_2 fixation and seed yield. Effects of plant density on the pattern of growth of medics are well documented in the literature (Adem 1977; Silsbury *et al.* 1979; Squella 1992). Initially, growth is directly related to plant density, through an increase in the number of branches initiated by developing shoots. At later stages, however, the reverse occurs with growth being inversely related to density. Initial differences in the number of primary branches are increased through increased self pruning of branches, although this is not true at very low densities (Smith and Jordan 1994). In general, lower plant densities tend to increase individual plant sizes and rooting depth, but N_2 fixation is low per unit area (Shearer and Kohl 1986).

Herbicide application, defoliation and plant density also influence seed yield in pastures. In medic cultivars, considerable variation in components of seed yield exists even when end-of-season dry matter yields are similar (Cocks 1988, 1990; Carter 1989; Ababneh 1991).

Carter (1989) reported that a single mild defoliation (hay cut) at a height of 5-8 cm of the annual medics, Paraggio and Jemalong at the start of flowering had little effect on seed yield, whereas Tow and Al Alkailah (1981) reported that severe defoliation to a height of 1-2 cm at early flowering markedly reduced seed yield in Jemalong medic. According to Carter (1988), annual medic seed producers in South Australia try to increase seed yield by reducing excessive vegetative growth prior to flowering using low rates of herbicides. It would therefore seem important to investigate the extent to which low and recommended rates of herbicides can affect seed production in medic pastures.

While herbicides offer a means of controlling weeds in pastures, related adverse effects of reduced dry matter and seed yields and lower rates of N_2 fixation, require that alternative means of weed control are considered. In the experiment described here the effects of mechanical defoliation on nitrogen fixation, dry matter production and seed yields of Paraggio barrel medic sown at three rates were compared with the effects of herbicide application.

5.1.2 MATERIAL AND METHODS

5.1.2.1 Location

The experiment was conducted at the Waite Agricultural Research Institute (hereafter referred to as Waite Institute), Adelaide, South Australia during 1993 (Plate 5.1.1).

Plate 5.1.1 General view of the field experiment at Waite Institute, photographed on 4 August, 1993.

Rainfall and temperature for the year are shown in Table 5.1.1. The soil of the experiment site was classified as Urrbrae loam, a red-brown earth. It is a silty clay loam with 32.5% clay, 57% silt and 10% sand (Williams 1981). The soil is acidic at the surface (pH 5.50 - 5.87 in 0.01 M $CaCl_2$) and becomes alkaline with depth. Percentage total N, available inorganic N and $\delta^{15}N$ (inorganic N) measured in the 0 - 10 cm depth of soil from different replicates were 0.11%, 9.48 (mg/kg soil) and 6.2‰ respectively. A 40 m $\times$ 30 m area which had previously been sown to cereal, was selected for the experiment. This area was chosen because of the uniformity of cereal yields in the previous year.

Table 5.1.1 Rainfall and temperature data for the Waite Agricultural Research Institute during 1993 and 1994.

Month	1993			1994		
	Rainfall (mm)	Temperature (°C)		Rainfall (mm)	Temperature (°C)	
		Max.	Min.		Max.	Min.
Jan	31.4	27.5	16.6	44.4	25.1	14.8
Feb	40.0	27.4	17.0	16.0	27.1	16.1
Mar	18.8	25.1	15.3	0.0	26.3	15.4
Apr	1.4	24.2	14.1	16.0	22.0	13.3
May	49.4	19.1	11.3	32.6	18.3	10.8
Jun	44.8	14.6	8.3	81.0	15.4	9.7
Jul	74.8	14.9	9.1	49.6	15.9	8.6
Aug	59.8	17.5	10.3	19.4	14.9	7.9
Sept	75.2	18.2	10.1	31.6	16.5	9.1
Oct	77.6	19.9	11.4	49.4	20.8	12.0
Nov	23.0	24.2	13.4	50.0	21.8	12.5
Dec	76.0	24.1	14.8	9.2	28.4	16.8
Total	**572.2**			**399.2**		

5.1.2.2 Land preparation and experimental design

The site was cleared of wheat stubble and limed at the rate of 5 tons/ha. Superphosphate containing trace elements-Mo, Zn, Co, Cu was applied at 250 kg/ha on 20 March, 1993. Pre-sowing irrigation was used to stimulate weed emergence after which weeds were removed with cultivation.

The experiment was a split-plot design with sowing rate as the main plot and three defoliation practices, one herbicide treatment and a control as sub plots. These were completely randomised in 4 blocks. The main plots measured 2m × 25m and were split into five sub-plots of 2m × 5m. A distance of 2m was kept between replicates and 1m between main plots (Figure 5.1.1).

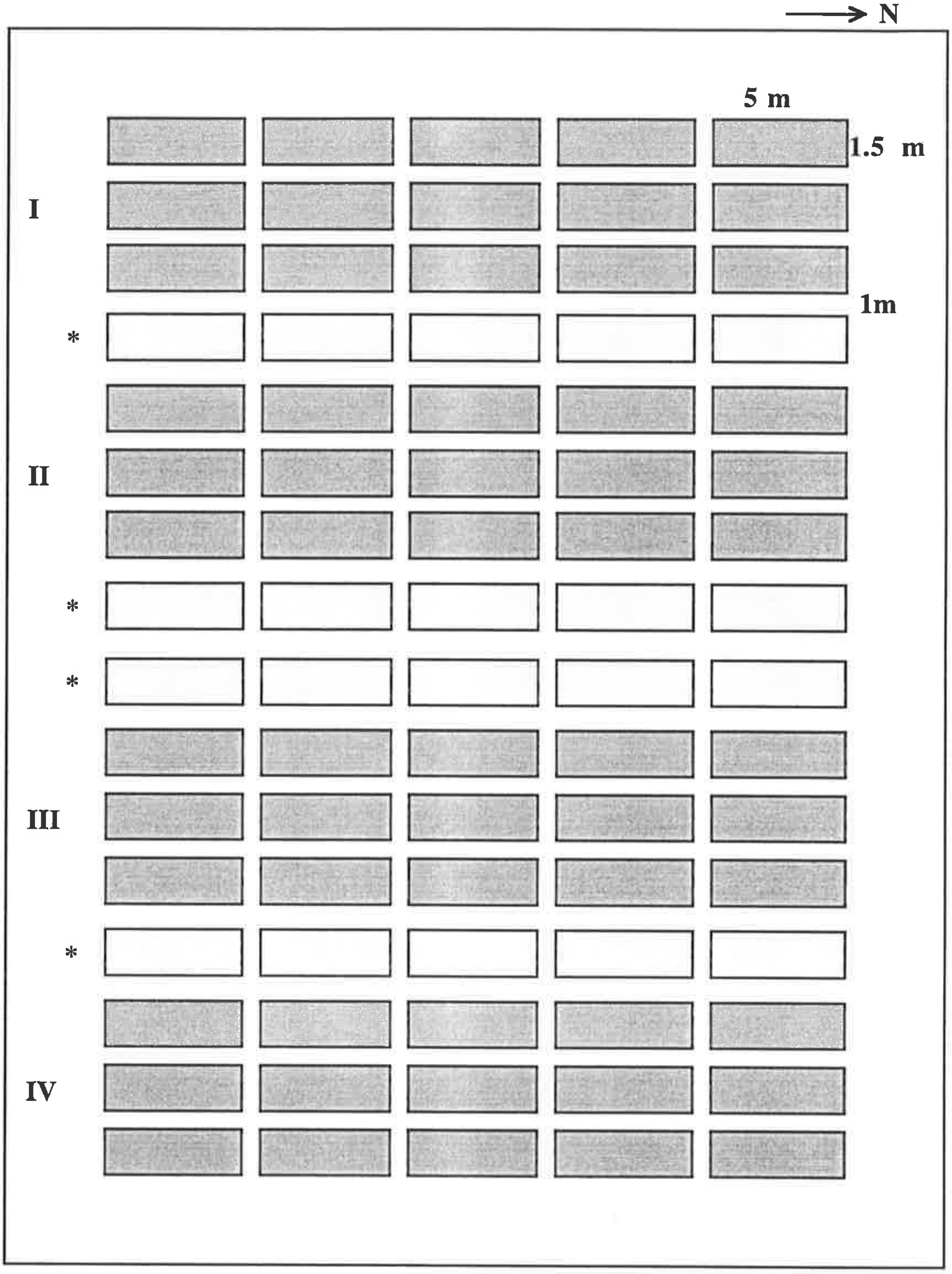


Figure 5.1.1 **Field layout of Experiments 5 and 6 showing the split plot design, using sowing rates in drill plots as main plots within Blocks I, II, III and IV also the five defoliation and herbicide treatments as subplots. (* Four main plots used for Experiment 6).**

5.1.2.3 Sowing

The experimental area was rolled and harrowed prior to sowing on 3 June 1993. Seeds of *Medicago truncatula* cv. Paraggio were inoculated with *Rhizobium meliloti* in peat culture (inoculant group A) just before sowing by coating them with a recommended amount of the inoculant as a sugar+water slurry and allowing time to dry. The inoculated seeds were sown using a cone seeder mounted an a 10-row-drill with row spacing of 15 cm. Some annual ryegrass (*Lolium rigidum*) seed was sown at the corner of plots to provide reference plants for N fixation measurements. The experiment was lightly harrowed after sowing to ensure good coverage of the seed.

5.1.2.4 Treatments

Treatments consisted of three sowing rates of *Medicago truncatula* cv. Paraggio (20, 100 and 500 kg/ha based on pure germinating seed) as main plots, three levels of defoliation (early defoliation, late defoliation and one double defoliation (early+late), 2,4-DB herbicide applied at a rate of 2.8 L/ha together with a non-defoliated, unsprayed control making five subplots.

The defoliation was imposed by mowing at a height of 5 cm above the ground. The early mowing was at the 10 leaf stage on 6 August and the late mowing at the beginning of flowering on 3 September 1993. The early+late mowing treatment was a combination of the two single treatments.

The herbicide was mixed with Agral 600 as a wetting agent and sprayed using a boom-sprayer at a constant height of 40 cm above plant tops. The herbicide was sprayed at the same time as the early defoliation treatment, i.e. 6 August.

5.1.2.5 Sampling and measurements

The medic plots were sampled for plant density, dry matter yield (shoots and roots), N_2 fixation, seed production, medic regeneration and soil nitrogen.

Dry matter yields, nitrogen fixed from the atmosphere and that derived from the soil by the legume were measured three months after sowing, when the *Rhizobium*-plant symbiosis was presumed to be fully developed. From then on, sampling was carried out at monthly intervals until the end of the season.

Samples were taken four times during plant growth for plant density, dry matter yield and N_2 fixation. At each time of sampling, cuts were taken from two random quadrats (from the northern and southern halves of each plot) before and after each cut to estimate herbage removal. At the first two sampling occasions wire quadrats of 25 × 40 cm were used; and for the other two harvests, quadrats of 50 × 50 cm were used. Areas from where quadrat samples had been taken were marked by pegs to ensure that later sampling of herbage, roots or seed avoided these areas (Plate 5.1.2).

Plate 5.1.2 View of defoliated and sampled medic swards on 16 Sept. 1993

Plant density was estimated by harvesting plants just below ground level so that plant numbers could be counted within each quadrat. Dry matter was estimated by cutting plants at ground level, then harvested plants were dried at 85°C in a forced-draught dehydrator for 24 hours and weighed. At the same time as the dry matter sampling, a sample of 10 legume plants was cut close to each sampling quadrat to measure N_2 fixation. Reference annual ryegrass shoots were also collected from the same plot . The samples for N_2 fixation measurement were dried at 60°C in a forced-draught dehydrator for 24 hours. N_2 fixation was determined as described in Chapter 4 using the ^{15}N dilution technique.

Sampling for nodulated roots was undertaken on 1 November. Two soil cores including plant roots were taken at random from the north and south of each plot using a truck-mounted sampling tube (14.7 cm diameter, 30 cm depth) (Plate 5.1.3).

Plate 5.1.3 Soil coring for root sampling by a truck-mounted Fletcher hydraulic driven rig.

The cores were processed by careful hand washing and separating the roots from the soil as described in Chapter 4. The cleaned roots were used for estimating N_2 fixation and nodule mass after drying at 60 °C in a forced-draught dehydrator for 24 hours (Plate 5.1.4).

Plate 5.1.4 Cleaned roots and nodules for estimating root dry matter yield and nodule mass.

A seed harvest was made between 1 and 4 December 1993 using a ring quadrat of 28 cm diameter to define the sample area. A north and a south sample were taken from each plot in areas where herbage samples had not already been taken. Dry pasture residues and pods that had fallen on the ground were collected in separate bags. A hand broom was used to collect the above-ground samples. All pods were carefully separated from dry pasture residues and cleaned free of soil in Genclean® (1,1,1 trichloroethane) (Carter *et al* 1977). The pod samples were then oven dried at 40°C in a forced-draught dehydrator for 24 hours and weighed. To obtain seed samples, a medic/sub clover seed thresher was used to separate seed from husks. Seed yield and components determined were pod number/m^2, mean pod weight (mg), seed number per pod, total seed yield (kg/ha), mean seed weight (mg) and seed:pod ratio (%).

Regeneration of medic seedlings was estimated in the experimental area, using two random quadrats (25 cm × 40 cm) taken from the north and south of each of the plots. Seedling populations were counted on December 4,1993; January 25 and April 5,1994 following natural rainfall events.

Soil samples were taken for total N% , available N and $\delta^{15}N$ measurements on June 6, August 8 and November 2,1993 from the corners and middle of the experimental area. Soil sampling and soil analysis procedures were as described in Chapter 4.

5.1.2.6 Statistical analyses

Data were analysed using a split-plot analysis. Natural logarithm transformations were made where the data was not homogeneous. Each sowing rate was analysed separately so that treatment differences within medic densities could be measured. Overall analyses were also made to detect sowing rate effects and sowing rate × defoliation and herbicide interactions for the parameters measured. Least significant difference procedures were used for comparison of means.

5.1.3 RESULTS

5.1.3.1 Plant establishment and density

Seedlings emerged one week after sowing. This was less than expected (Table 5.1.2).

Table 5.1.2 Seedling emergence of Paraggio barrel medic 7 days after sowing.

Sowing rate (kg/ha)	Expected plants/m^2	Actual plants/m^2	Emergence (%)
20	381	233	61
100	1905	871	46
500	9525	4011	42

The discrepancy in emergence may have been due to lifting of the soil crust caused by the large number of seedlings emerging at the same time on the higher-sowing-rate plots which caused desiccation of seedlings. Mean plant numbers over all treatments were fairly uniform at the 20 kg/ha sowing rate, but declined through self-thinning at 100 kg/ha sowing rate and particularly at 500 kg/ha sowing rate throughout the growing season (Fig. 5.1.1). At the higher sowing rates (100 and 500 kg/ha), plants in late-defoliated plots did not recover from defoliation and all medic plants died.

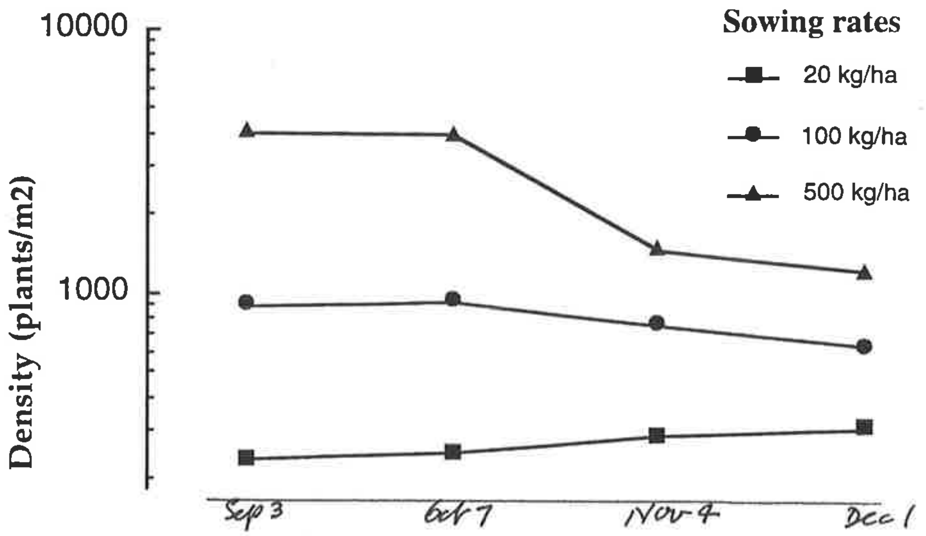


Figure 5.1.1 **Effects of three sowing rates on plant density of Paraggio medic during the growing season. (log transformation). Note: The above data exclude the zero values for 100 and 500 kg/ha sowing rates following late defoliation.**

5.1.3.2 Dry matter yield

Shoots: A summary of the main effects of sowing rate, defoliation and herbicide application and their interactions is presented in Table 5.1.3 and shows a highly significant sowing rate and defoliation and herbicide effect throughout the sampling period. Because late defoliation (3 September) killed the Paraggio medic grown on the medium and high density plots there was no herbage to harvest, so these treatments were excluded from the statistical analyses. Consequently the Sowing rate × Defoliation and herbicide interaction is shown as non-significant in Table 5.1.3. Yet, in reality, there was a marked interaction between sowing rates and defoliation as shown in Figure 5.1.2. The low sowing rate (low density) stand of medic, which was more prostrate, withstood the impact of defoliation whereas the medic at higher density was more erect and lost all leaves and growing points at the late defoliation, hence the medic plants died.

Table 5.1.3 **Summary of analyses of variance carried out on shoot dry matter yield of Paraggio barrel medic.**

Source of variation	Harvests			
	Sep 3	Oct 7	Nov 4	Dec 1
Sowing rates	***	***	***	*
Defoliation and herbicide treatments	***	***	***	***
Sowing rates × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Visual assessment indicated that lower stand density promoted primary branches which led to increasing leaf area and thus light interception by medic plants (Plate 5.1.5).

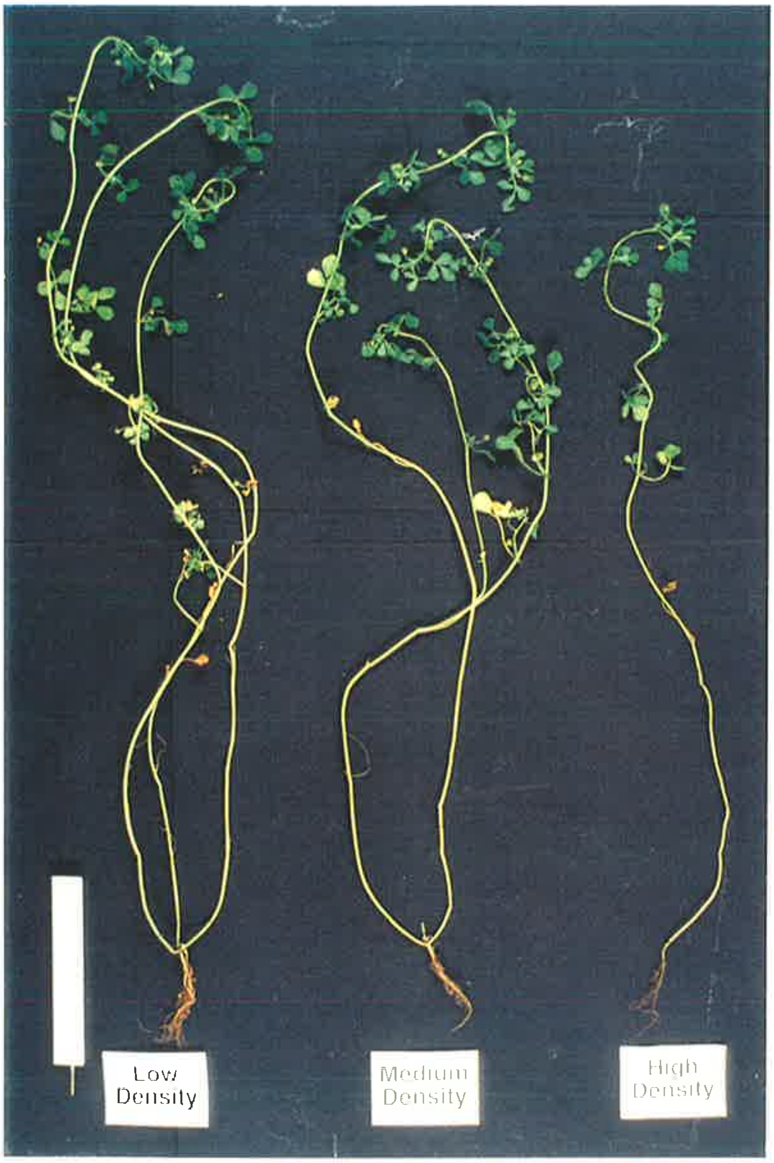
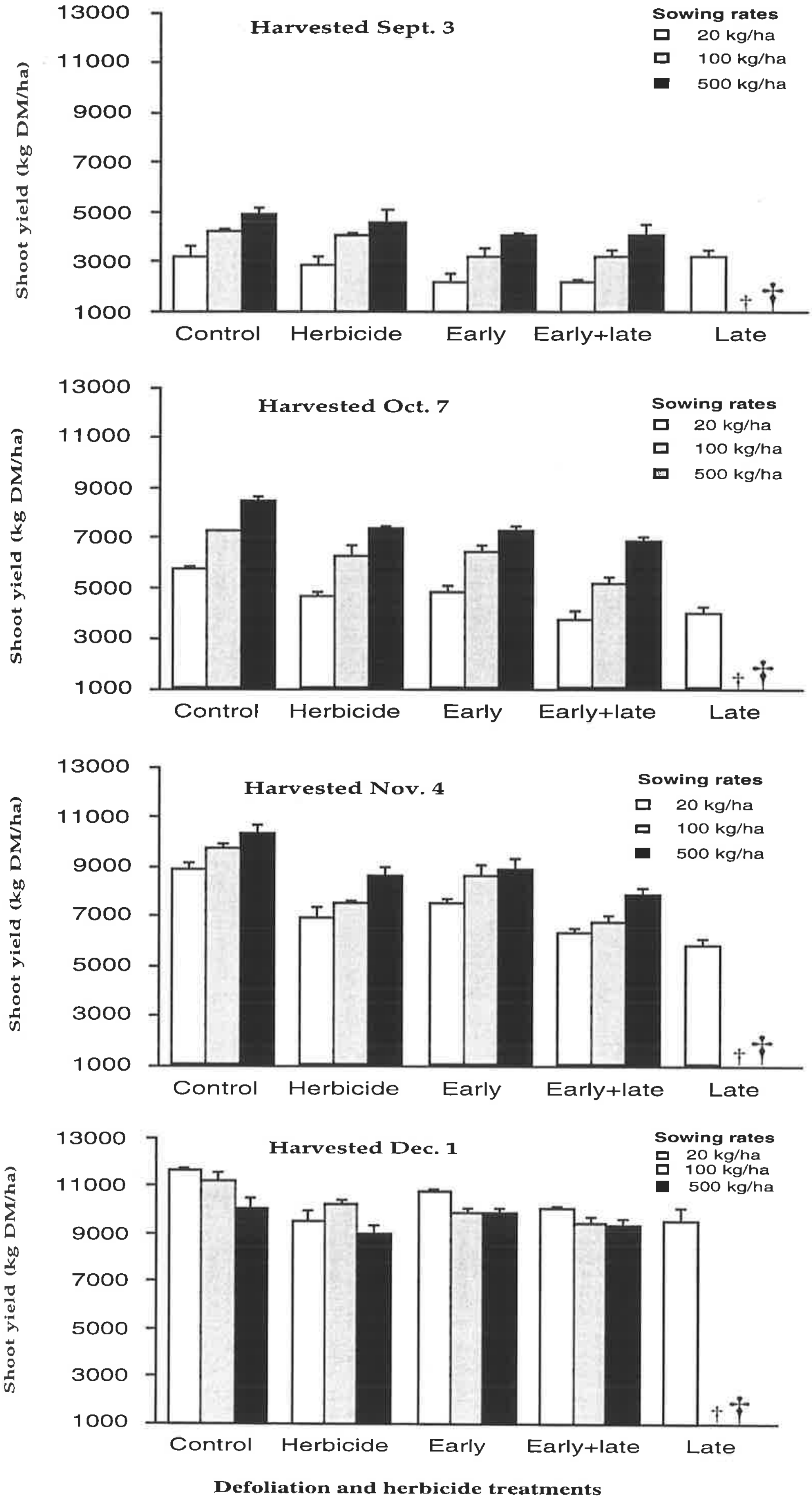


Plate 5.1.5 Effects of sowing rates on promotion of primary branches in Paraggio barrel medic.

Figure 5.1.2 The effects of sowing rate (plant density), defoliation and herbicide treatments on medic yield on four harvest occasions. (Values represent the mean of four replicates, the bars indicate S.E., † and † represent trace amounts of material from 100 and 500 kg/ha sowing rate plots respectively remaining after late defoliation which was not enough for sampling).


Harvested Sept. 3
Sowing rates
20 kg/ha
100 kg/ha
500 kg/ha
Shoot yield (kg DM/ha)
13000
11000
9000
7000
5000
3000
1000
Control
Herbicide
Early
Early+late
Late
Harvested Oct. 7
Harvested Nov. 4
Harvested Dec. 1
Defoliation and herbicide treatments

All defoliation treatments gave cumulative yields significantly lower than that of the undefoliated control, although regrowth occurred in the swards after defoliation in all treatments, except in the late defoliation treatments (Table 5.1.4). The early+late defoliation on August 5 and September 3 respectively, also reduced cumulative yields significantly (P<.05) compared to controls and the early defoliation treatment.

Table 5.1.4 Yield (kg DM/ha) of medic forage removed at defoliation, and total cumulative yield of Paraggio medic on September 3 and October 7,1993.

Treatments	Removed at defoliation		Yield		Total cumulative yield	
	Aug 5	Sept 3	Sept 3	Oct 7	Sept 3	Oct 7
20 kg/ha sowing rate						
Control	-	-	3291	4782	3291	5780
Early defoliation	225	-	1986	4742	2211	4742
Early+late defoliation	215	1935	2036	1810	2251	3746
Late defoliation	-	1876	3293	2125	3293	4001
LSD (5%)					956	993
100 kg/ha sowing rate						
Control	-	-	4234	8224	4234	7274
Early defoliation	690	-	2563	6516	3253	6516
Early+late defoliation	622	2090	2562	3100	3184	5190
Late defoliation	-	3583	4183	tr	4183	tr
LSD (5%)					671	753
500 kg/ha sowing rate						
Control	-	-	4969	8497	4969	8497
Early defoliation	972	-	3120	7290	4092	7290
Early+late defoliation	1126	1967	2980	4889	4106	6856
Late defoliation	-	4283	5042	tr	5042	tr
LSD (5%)					753	755

tr = trace amount of plant remaining after defoliation, which was not enough for sampling and excluded from statistical analyses.

Figure 5.1.3 shows the effect of defoliation on herbage yields. The slope of these log yield curves show the higher relative growth rates of defoliated treatments compared to controls.

Recovery of the swards and the curve fit for all defoliation treatments at all sowing rates were similar at the final harvest.

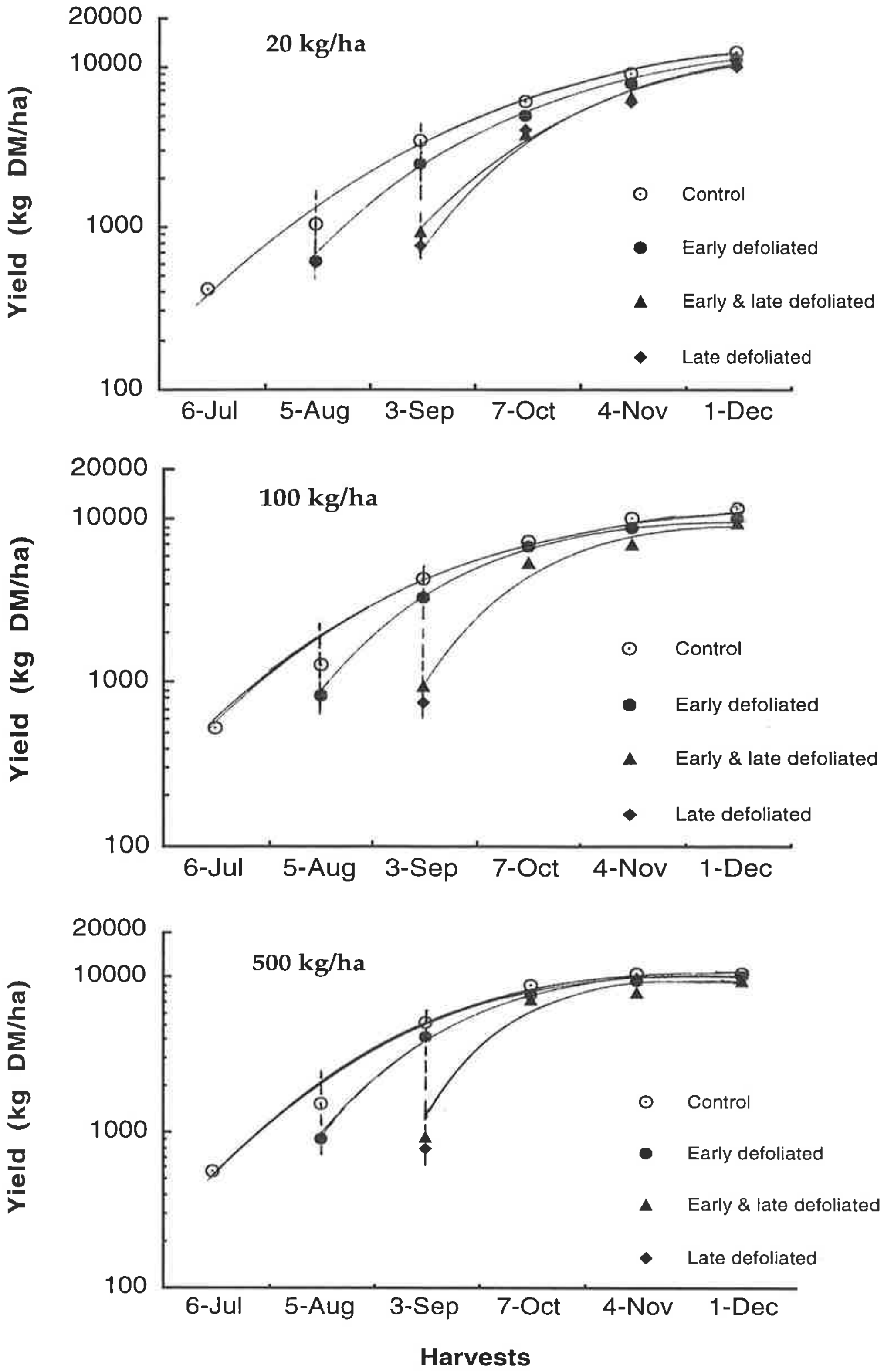


Figure 5.1.3 **Effects of defoliation on herbage yield of Paraggio medic sown at 20 kg/ha, 100 kg/ha and 500 kg/ha. Yield data have been transformed to logarithms.**

Symptoms of herbicide injury (change in leaf morphology and scorching of leaves) resulting in rolled leaflet margins, which later changed to a strap-like appearance, were observed on herbicide-treated plants within 7 days of herbicide application. Evidence of growth retardation was also observed (Plate 5.1.6).

Plate 5.1.6 Effects of herbicide application on plant vigour and its effect on the leaf systems. A = Herbicide sprayed, B = Unsprayed control.

Shoot dry matter yields (for all herbicide/defoliation treatments) at the first harvest were lowest at the lowest sowing rate (20 kg/ha) and highest in the highest sowing rate swards (500 kg/ha) (Plate 5.1.7). Shoot yield then increased rapidly at the lowest sowing rates so that by the final harvest dry matter yield was greater at the low (20 kg/ha) and least at highest (500 kg/ha) sowing rates (Table 5.1.5).

Plate 5.1.7 Effects of sowing rates on medic plant growth two months after sowing.

Table 5.1.5 Overall effects of defoliation and herbicide treatments on shoot yields of Paraggio barrel medic sown at three sowing rates.

	Shoot yield (kg DM/ha) at harvests on:			
Sowing rates (kg/ha)	Sept 3	Oct 7	Nov 4	Dec 1
20	2789	4615	7135	10346
100	3774	5048	6545	8185†
500	4563	6002	7172	7690†
LSD (5%)	337	187	327	376

† Data for late defoliation plots, where plants died, is excluded.

The yield and distribution of dry matter at monthly intervals for control and treated plants is shown in Table 5.1.6. Yield of all treatments other than late-defoliated swards sown at 100 kg/ha and 500 kg/ha increased throughout the growing season. Plants did not recover from the single late-defoliation of swards sown at 100 kg/ha and 500 kg/ha and hence further sampling of these treatments was discontinued.

At the final harvest, dry matter yields produced by the early defoliation treatment at all three sowing rates were slightly less than the control (but not significantly). In the 20 kg/ha sowing, however, a significant reduction in the herbicide treatment compared to the control and early defoliation treatment occurred. All other treatments reduced DM yields relative to the control (significantly so in most cases). In contrast the herbicide-sprayed plants sown at 100 kg/ha gave higher shoot yields than the early+late defoliation treatments, while in the 500 kg/ha swards there were no differences in the shoot yields between treatments.

Table 5.1.6 Effects of defoliation (early on Aug.6, late on Sept.3 and early+late on both Aug.6 and Sept.3) and herbicide application (2,4-DB, 2.8 L/ha sprayed on Aug.6) on dry matter yields (kg/ha) of Paraggio barrel medic at three sowing rates at the Waite Institute in 1993.

Treatments	Yield (kg DM/ha) at harvests on:			
	Sept 3	Oct 7	Nov 4	Dec 1
20 kg/ha sowing rate				
Control	3291	5780	8934	11680
Early defoliation	2211	4742	7571	10790
Early+late defoliation	2251	3746	6353	10120
Late defoliation	3293	4001	5862	9604
Herbicide application	2898	4708	6956	9535
LSD (%)	862	866	783	1007
100 kg/ha sowing rate				
Control	4234	7274	9770	11184
Early defoliation	3253	6516	8657	10584
Early+late defoliation	3184	5190	6734	9460
Late defoliation	4183	tr	tr	tr
Herbicide application	4016	6259	7562	10295
LSD (%)	617	869	898	798
500 kg/ha sowing rate				
Control	4969	8497	10341	10079
Early defoliation	4092	7290	8969	9963
Early+late defoliation	4106	6856	7883	9398
Late defoliation	5042	tr	tr	tr
Herbicide application	4607	7364	8669	9012
LSD (%)	808	616	1132	NS

tr = trace amount of plant remained after the defoliation, which was not enough for sampling and excluded from statistical analyses.

Roots: Contribution of roots to total pasture DM was low and did not show any differences in root DM between the three sowing rates when sampled on November 1. However, root weight on a plant basis was significantly different in swards sown at the three rates. A summary of the main effects on root parameters of sowing rate, defoliation and herbicide treatments and their interactions are shown in Table 5.1.7. A highly significant Sowing rate × Defoliation and herbicide interaction was observed for rooting depth only. Both sowing rate and defoliation and herbicide exerted significant effects on root dry matter per plant and root depth. The interaction of sowing rates, defoliations and herbicide on root weight is also shown in Figure 5.1.4.

Table 5.1.7 Summary of analyses of variance of data on root dry matter, rooting depth and nodule dry weight of Paraggio barrel medic

Source of variation	Root dry matter	Root depth	Nodule dry matter
Sowing rates	***	***	*
Defoliation and herbicide	*	**	NS
Sowing rates × Defoliation and herbicide	NS	***	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

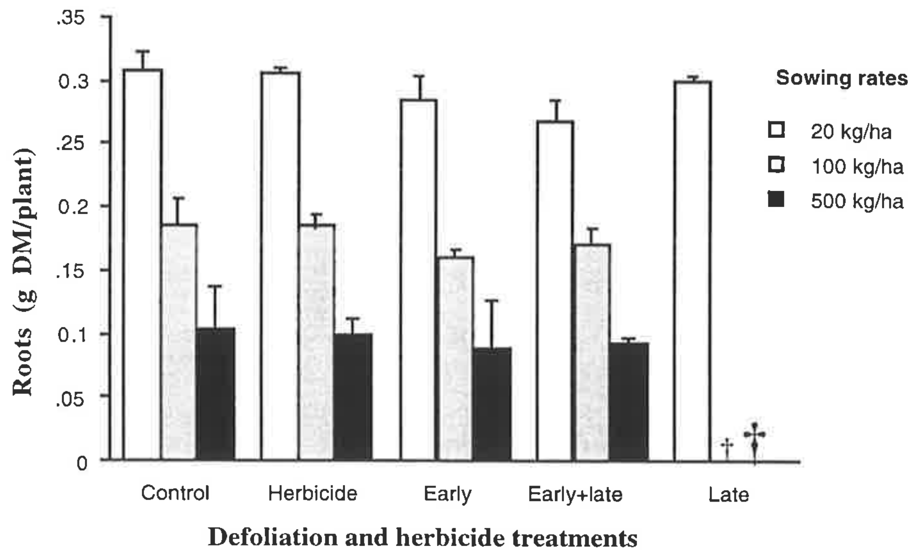


Figure 5.1.4 The effects of sowing rate, defoliations and herbicide on root dry matter yields of Paraggio barrel medic. (Values represent the mean of four replicates while the bars indicate S.E., † and † represent trace amounts of material from 100 and 500 kg/ha sowing rate plots respectively remaining after late defoliation which was not enough for sampling).

Generally root dry matter per plant and rooting depth were lower at the higher sowing rates (Table 5.1.8).

Table 5.1.8 Overall effects of sowing rate on root dry matter, rooting depth and nodule dry weight in Paraggio barrel medic (average of defoliation and herbicide treatments).

Sowing rate (kg/ha)	Root dry matter (g/plant)	Root depth (cm)	Nodule dry weight (mg/plant)
20	0.29	14.5	29
100	0.18	8.9	26
500	0.10	6.8	17
LSD (5%)	0.02	1.8	5.7

Root dry matter in controls did not differ significantly from that in defoliated and herbicide-treated plants (Table 5.1.9). Rooting depth of plants grown at the low sowing rate (20 kg/ha) was significantly greater than those of plants grown at the higher sowing rates (100 and 500 kg/ha). Early+late defoliated plants had significantly less rooting depth than the other treatments.

Table 5.1.9 Effects of defoliation (early on Aug.6, late on Sept.3, early+late on both Aug.6 and Sept.3) and herbicide application (2,4-DB at 2.8 L/ha sprayed on Aug.6) on root dry matter, rooting depth and nodule dry weight of Paraggio barrel medic sown at three rates.

Treatments	Root dry matter (g/plant)	Root depth (cm)	Nodule dry weight (mg/plant)
20 kg/ha sowing rate			
Control	0.31	16.0	36.2
Early defoliation	0.28	15.8	30.1
Early +late defoliation	0.27	8.8	25.5
Late defoliation	0.30	16.7	28.8
Herbicide application	0.31	15.3	24.8
LSD (%)	NS	3.1	8.6
100 kg/ha sowing rate			
Control	0.19	10.0	26.8
Early defoliation	0.16	9.5	28.4
Early +late defoliation	0.17	8.2	25.9
Late defoliation	tr	tr	tr
Herbicide application	0.19	8.0	24.3
LSD (%)	NS	NS	NS
500 kg/ha sowing rate			
Control	0.11	6.0	17.9
Early defoliation	0.11	6.0	17.8
Early +late defoliation	0.09	7.3	15.9
Late defoliation	tr	tr	tr
Herbicide application	0.10	7.8	16.7
LSD (%)	NS	NS	NS

5.1.3.3 Nodules

There were no significant interactions between sowing rates and defoliation and herbicide treatment on nodule dry weight (Table 5.1.7). However, nodule weight was reduced significantly at the highest sowing rate (Table 5.1.8, Figure 5.1.5). Nodule dry weights of medic sown at 20 kg/ha and sprayed with herbicide were significantly lower than the control plants and were lower than the nodule dry weights of the defoliated medic plants. At the lowest sowing rate, only the early defoliation combined with late defoliation and the herbicide treatments significantly reduced nodule weight per plant compared to the controls (Table 5.1.9).

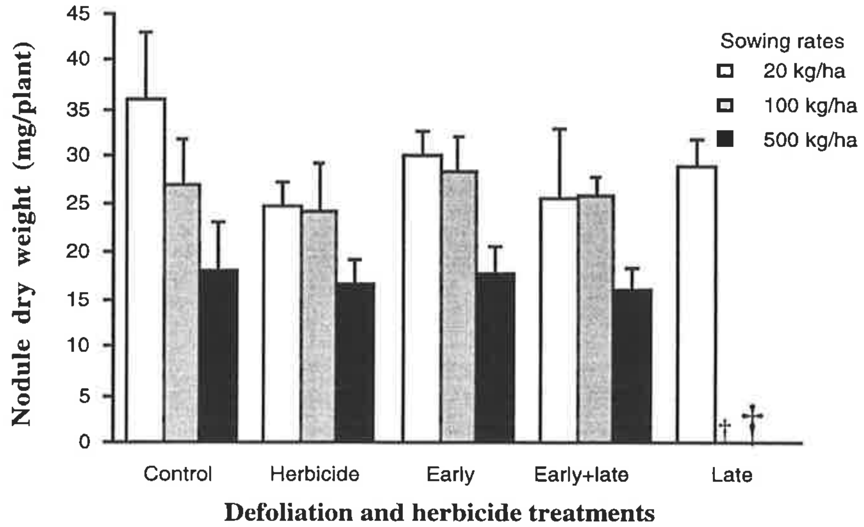


Figure 5.1.5 Effects of sowing rate and defoliation and herbicide treatments on nodule dry weight of Paraggio barrel medic. (Values represent the mean of four replicates while the bars indicate S.E., † and † represent trace amounts of material from 100 and 500 kg/ha sowing rate plots respectively remaining after late defoliation which was not enough for sampling).

5.1.3.4 Nitrogen in shoots

***Percentage N and total N*:** There were no significant interactions between sowing rate and the defoliation and herbicide treatment on percentage N and total plant N (Tables 5.1.10 and 5.1.11).

Table 5.1.10 Summary of analyses of variance on percentage N of Paraggio barrel medic.

	Harvests			
Source of variation	Sept 3	Oct 7	Nov 4	Dec 1
Sowing rate	*	NS	NS	NS
Defoliation and herbicide	***	***	***	NS
Sowing rate × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Table 5.1.11 Summary of analyses of variance on N yield of Paraggio barrel medic.

Source of variation	Harvests			
	Sept 3	Oct 7	Nov 4	Dec 1
Sowing rate	***	***	***	NS
Defoliation and herbicide	NS	***	***	NS
Sowing rates × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Over all defoliation and herbicide treatments, percentage N was significantly reduced at the 20 kg/ha sowing rate compared to the other treatments at the first harvest only (Table 5.1.12). Percentage nitrogen in the plants increased with sowing rate at the first harvest significantly so for plants sown at 500 kg/ha compared to 20 kg/ha. Percentage nitrogen was also significantly higher in younger plants but was progressively reduced to about half of the initial value in mature plants (Table 5.1.12). However, total plant N increased with increasing sowing rate until the final harvest when the differences disappeared (Table 5.1.13). The trend was similar to that of total plant DM.

Table 5.1.12 Overall effects of defoliation and herbicide treatments on percentage N in Paraggio barrel medic sown at three rates.

Sowing rates (kg/ha)	% N at harvests on:				LSD (5%)
	Sept 3	Oct 7	Nov 4	Dec 1	
20	3.9	2.7	2.2	2.0	0.27
100	4.0	2.6	2.2	2.1	0.32
500	4.1	2.7	2.2	2.2	0.31
LSD (5%)	0.17	NS	NS	NS	

Table 5.1.13 Overall effects of defoliation and herbicide treatments on N yield in Paraggio barrel medic sown at three rates.

Sowing rates (kg/ha)	N yield (kg N/ha) at harvests on:			
	Sept 3	Oct 7	Nov 4	Dec 1
20	108	121	154	208
100	148	161	174	211
500	187	204	196	199
LSD (5%)	15	15	5	NS

Defoliation generally increased % N in all swards irrespective of sowing rate. This was especially marked for the early+late defoliation treatments (Table 5.1.14). In the herbicide-sprayed plots there were no significant differences in % N when compared with controls.

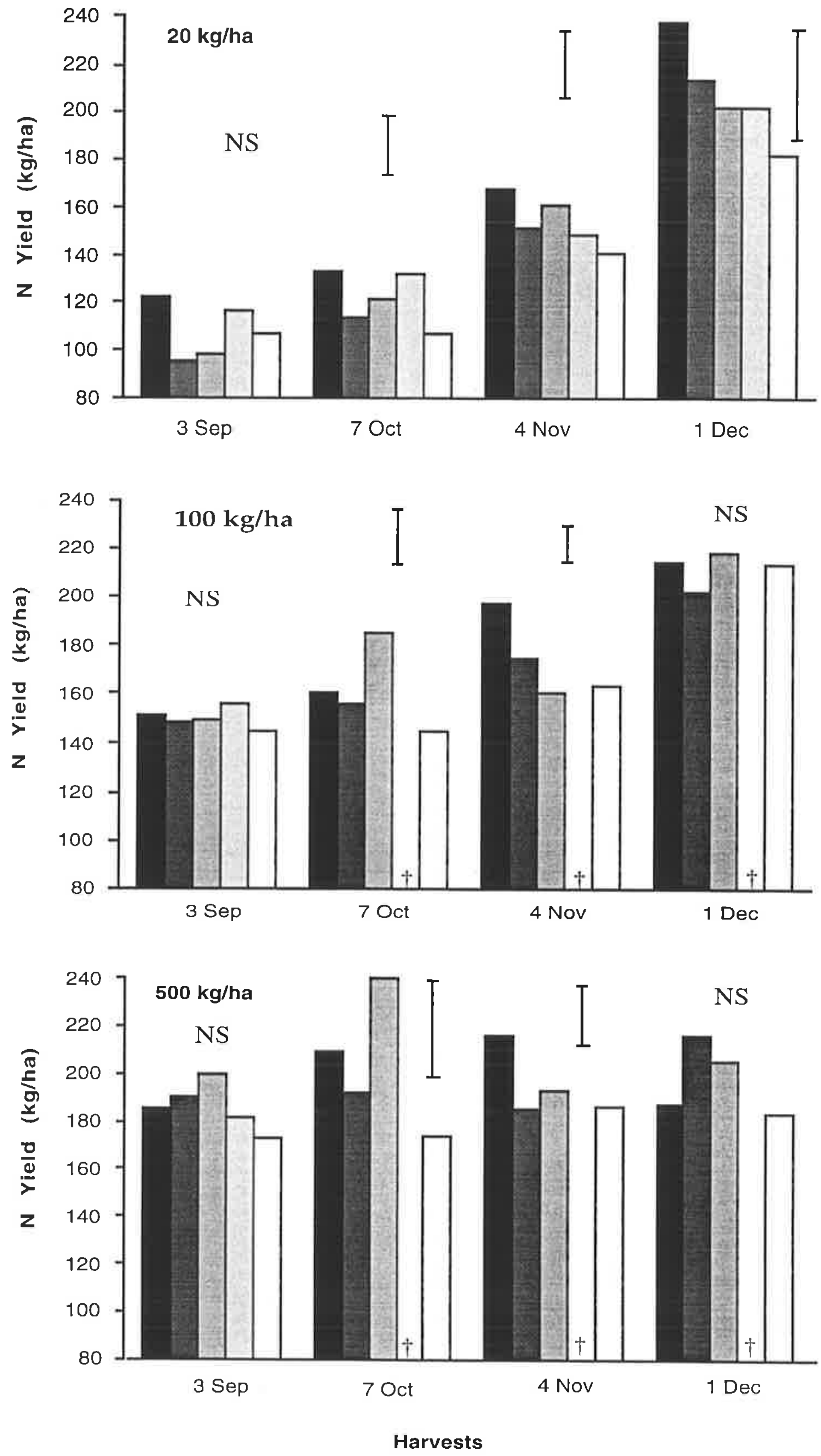
Table 5.1.14 Effects of defoliation (early on Aug.6, late on Sept.3, early+late on both Aug.6 and Sept.3) and herbicide application (2,4-DB at 2.8 L/ha sprayed on Aug.6) on % N of Paraggio barrel medic at three sowing rates.

Treatments	% N at harvests on:			
	Sept 3	Oct 7	Nov 4	Dec 1
20 kg/ha sowing rate				
Control	3.6	2.3	1.9	2.0
Early defoliation	4.4	2.3	2.0	2.0
Early +late defoliation	4.4	3.2	2.5	2.0
Late defoliation	3.6	3.3	2.5	2.1
Herbicide application	3.7	2.3	2.0	1.9
LSD (5%)	0.65	0.31	0.22	NS
100 kg/ha sowing rate				
Control	3.6	2.2	2.0	1.9
Early defoliation	4.6	2.4	2.0	2.0
Early +late defoliation	4.7	3.6	2.4	2.3
Late defoliation	3.7	tr	tr	tr
Herbicide application	3.6	2.3	2.2	2.1
LSD (5%)	0.50	0.13	0.21	0.23
500 kg/ha sowing rate				
Control	3.7	2.5	2.1	1.9
Early defoliation	4.6	2.6	2.1	2.2
Early +late defoliation	4.9	3.5	2.4	2.2
Late defoliation	3.6	tr	tr	tr
Herbicide application	3.7	2.4	2.2	2.0
LSD (5%)	0.37	0.31	0.16	NS

tr = trace amount of plant remained after the defoliation, which was not enough for sampling and was excluded from statistical analyses.

The application of herbicide on all but one occasion (Nov. harvest, medic sown at 100 kg/ha) resulted in a sward of lower total nitrogen than that of control plots. However, the difference was only significant in swards sown at 20 kg/ha at all harvests except the first. At the higher sowing rates, significant differences occurred only at the Nov 4 harvest. At the 100 and 500 kg/ha sowing rates, significant differences were also observed between the herbicide and early+late defoliated treatments at the second harvest only (Figure 5.1.6).

Figure 5.1.6 **Effects of defoliation and herbicide on the total N yield of Paraggio barrel medic sown at three rates (20, 100 and 500 kg/ha).** (Bars indicate LSD (5%), while † represents trace amounts of material remaining after late defoliation which was not enough for sampling).



■ = Control, ■ = Early defoliation, ▨ = Early+late defoliation, □ = Late defoliation, □ = Herbicide application).

Proportion of fixed N (P fix): The distribution of symbiotically-fixed N_2 expressed as the proportion of the medic N due to fixation (P fix). The interaction between sowing rate and defoliation and herbicide was not significant for P fix at any harvest (Table 5.1.15). However, the effects of defoliation and herbicide were significant at all harvests except at the first harvest. A sowing rate effect on P fix was significant at the 3rd and 4th harvests.

Table 5.1.15 Summary of analyses of variance on P fix data from Paraggio barrel medic.

Source of variation	Harvests			
	Sept 3	Oct 7	Nov 4	Dec 1
Sowing rate	NS	NS	**	*
Defoliation and herbicide	NS	*	***	**
Sowing rates × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Over all defoliation and herbicide treatments, P fix decreased throughout the growing season (Table 5.1.16).

Table 5.1.16 Overall effects of defoliation and herbicide treatments on P fix in Paraggio barrel medic sown at three rates.

Sowing rates	% P fix at harvests on:				LSD
(kg/ha)	3 Sep	7 Oct	4 Nov	1 Dec	(5%)
20	68	70	65	39	9
100	70	66	59	20	10
500	70	60	42	20	13
LSD (5%)	NS	NS	11	14	

As shown in Table 5.1.17, the estimated P fix was higher in defoliated plants (except in the late-defoliated plants at the medium and high sowing rates) than controls and herbicide-treated plants in the swards. For example, early-defoliated swards had higher P fix than herbicide-treated plants at the third harvest at low and medium sowing rates and at the final harvests at the high sowing rates. Also early+late defoliated plants had significantly higher P fix than herbicide-treated plants at the third harvest at the low sowing rate, at the final harvest at the medium sowing rate, and at all harvests except the first at the high sowing rate. The differences in P fix between defoliated plants and those of the control and herbicide-sprayed plants at most harvests at the high sowing rates, indicate a higher potential for nitrogen fixation in younger shoots relative to older shoots, probably because of new nodules replacing old nodules shed after defoliation.

Table 5.1.17 Effects of defoliation (early on Aug.6, late on Sept.3, early+late on both Aug.6 and Sept.3) and herbicide application (2,4-DB at 2.8 L/ha sprayed on Aug.6) on P fix (%) of Paraggio barrel medic at three sowing rates.

Treatments	P fix (%) at harvests on			
	Sept 3	Oct 7	Nov 4	Dec 1
20 kg/ha sowing rate				
Control	69	65	65	37
Early defoliation	71	76	70	46
Early +late defoliation	70	74	70	35
Late defoliation	67	69	64	46
Herbicide application	65	63	56	32
LSD (5%)	NS	NS	13	NS
100 kg/ha sowing rate				
Control	67	62	51	18
Early defoliation	75	78	73	29
Early +late defoliation	76	74	65	30
Late defoliation	66	tr	tr	tr
Herbicide application	65	52	48	10
LSD (5%)	NS	NS	22	15
500 kg/ha sowing rate				
Control	66	59	24	9
Early defoliation	73	66	39	32
Early +late defoliation	76	75	67	37
Late defoliation	67	tr	tr	tr
Herbicide application	65	41	37	3
LSD (5%)	NS	32	25	14

tr = trace amount of plant remained after the defoliation, which was not enough for sampling and was excluded from statistical analyses.

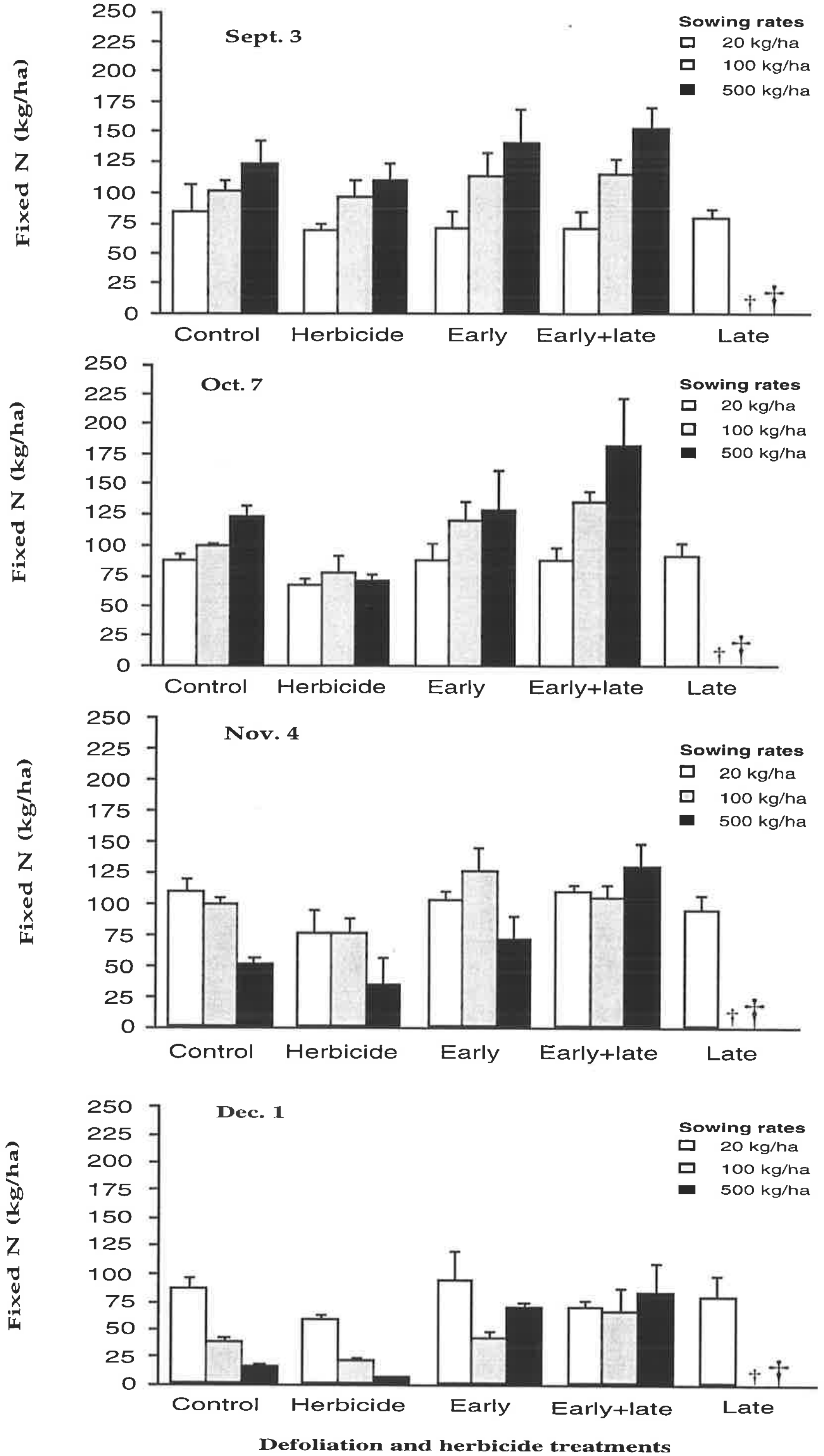
***N_2 fixation*:** The summary of the analyses of variance (Table 5.1.18) showed no significant interaction between sowing rate and defoliation and herbicide treatments except at the 3rd harvest for fixed N. Fixed nitrogen was consistently higher at the high sowing rates at the first harvest and had decreased at all sowing rates at the final harvest (Figure 5.1.7). At all harvests, the effect of sowing rate on N_2 fixed was significant except at the third harvest and the effect of defoliation and herbicide was significant except at the first harvest.

Table 5.1.18 Summary of analyses of variance on fixed N (kg/ha) from Paraggio barrel medic.

Source of variation	Harvests			
	Sept 3	Oct 7	Nov 4	Dec 1
Sowing rates	**	*	NS	*
Defoliation and/herbicide	NS	***	***	**
Sowing rates × Defoliation and herbicide	NS	NS	*	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Figure 5.1.7 **Effects of sowing rate, defoliation and herbicide on Fixed N from Paraggio barrel medic on Sept. 3, Oct. 7, Nov. 4 and Dec. 1.** (Values represent the mean of four replicates while the bars indicate the S.E., The † and ‡ represent trace amounts of material from 100 and 500 kg/ha sowing rate plots respectively remaining after late defoliation which was not enough for sampling).



Sept. 3
Oct. 7
Nov. 4
Dec. 1
Fixed N (kg/ha)
0
25
50
75
100
125
150
175
200
225
250
Sowing rates
20 kg/ha
100 kg/ha
500 kg/ha
Control
Herbicide
Early
Early+late
Late
†
Defoliation and herbicide treatments

The contribution of N from dinitrogen fixation was initially higher at the higher sowing rates. However, the pattern changed during the growing season, with higher contributions occurring at the low sowing rate at the final harvest (Table 5.1.19).

Table 5.1.19 Overall effects of defoliation and herbicide on the amount of nitrogen fixed from the atmosphere in Paraggio barrel medic sown at three rates.

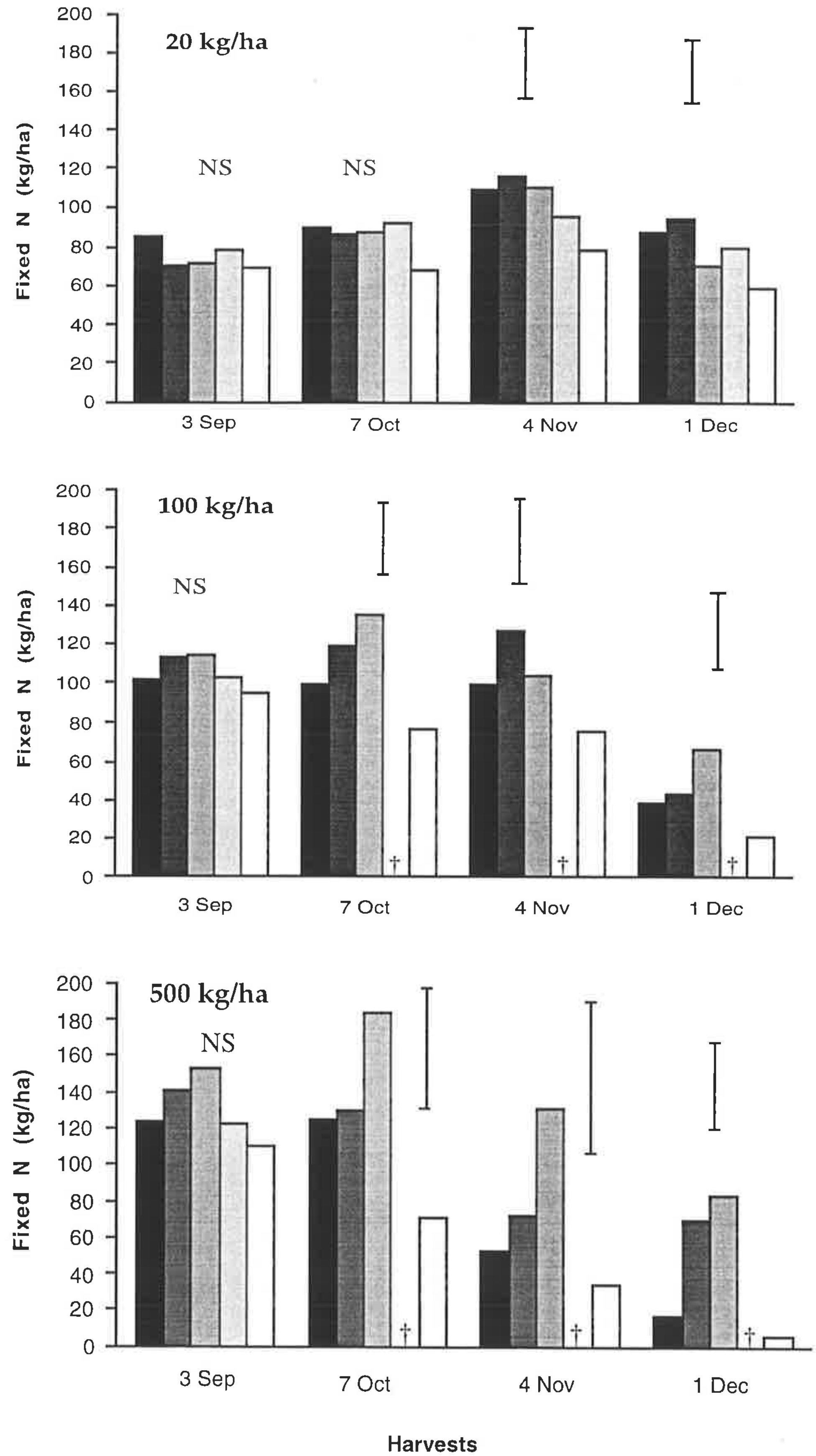
	N fixed from air (kg/ha) at harvests on:			
Sowing rates (kg/ha)	Sept 3	Oct 7	Nov 4	Dec 1
20	74	84	99	78
100	106	108	102	34
500	132	127	81	44
LSD (5%)	27	NS	NS	24

The amount of N_2 fixed in defoliated plants was slightly higher than that of controls at most harvests in all swards. In contrast, N_2 fixed in herbicide-sprayed plants was slightly lower than controls (Figure 5.1.8). Although the differences between treated plants were not statistically significant compared with controls, the positive effect of mechanical defoliation on nitrogen fixation in the medic was evident when the amount of N_2 fixed in defoliated and herbicide-treated plants were compared. For example, early-defoliated plants had significantly higher fixed N than herbicide-sprayed plants at the third and fourth harvests at low sowing rate, at the second and third harvest at medium sowing rate, and at the final harvest at the high sowing rate swards.

The source and partitioning of plant nitrogen is shown in Figure 5.1.9. Approximately three months after sowing (Sept 3) atmospheric N was the main source of N for plant growth. Dinitrogen fixation remained relatively constant until 4 November at the low sowing rate, but decreased sharply at the higher sowing rates.

The percentage of N in plants derived from available soil-N increased from about 30% at 3 September to the maximum (approximately 65% for low and 75% for high sowing rate) at final harvest, due to decreased N_2 fixation.

Figure 5.1.8 Effects of defoliation and herbicide on N fixed by Paraggio barrel medic sown at 20 kg/ha, 100 kg/ha and 500 kg/ha. (Bars indicate LSD (5%), while † represents trace amounts of material remaining after late defoliation which were not enough for sampling).



■ = Control, ■ = Early defoliation, ▣ = Early+late defoliation,
□ = Late defoliation, □ = Herbicide application).

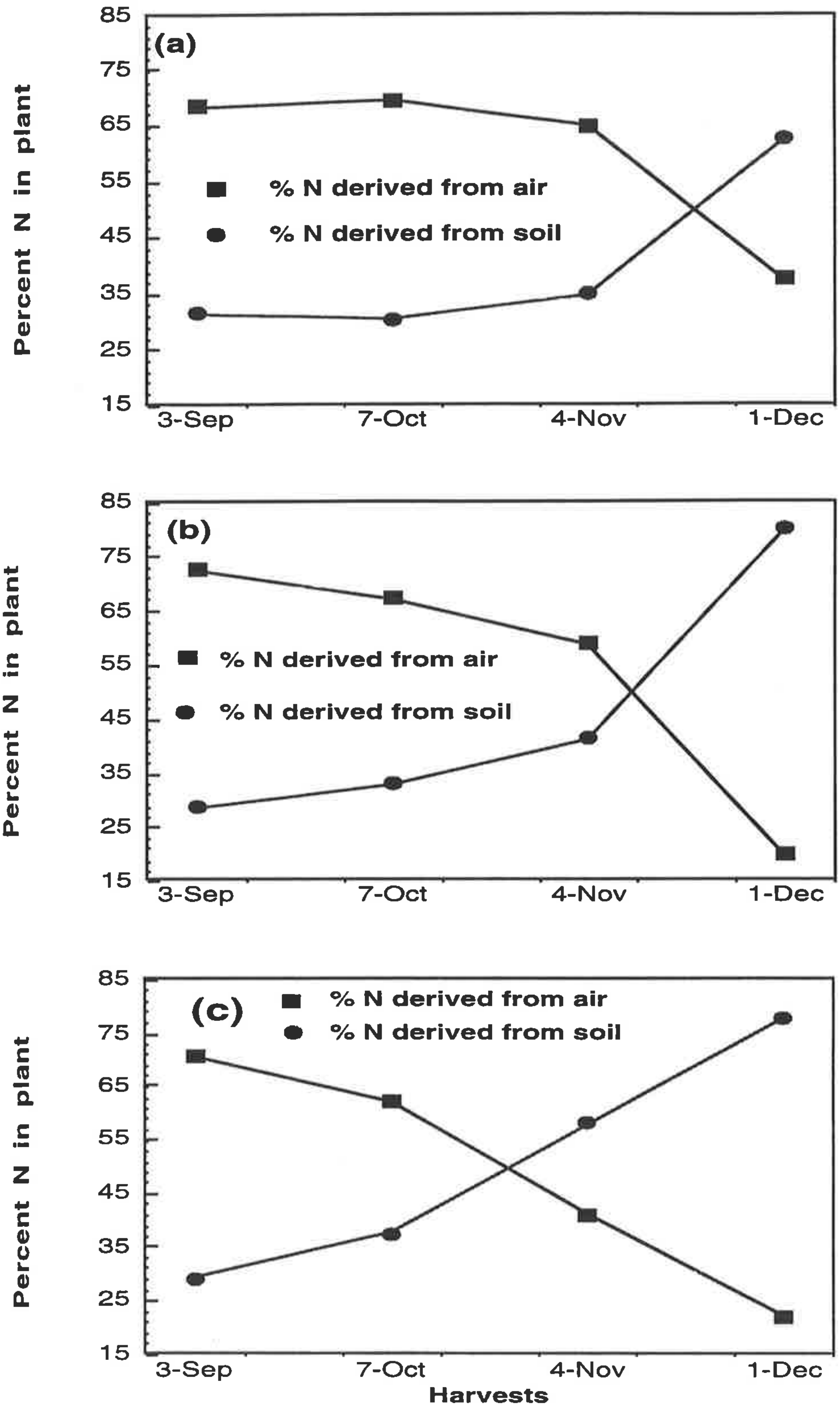


Figure 5.1.9 Seasonal proportions of N derived from soil and atmosphere in Paraggio medic sown at (a) 20, (b) 100 and (c) 500 kg/ha.

Correlations relating % P fix, plant dry matter yield, nodule dry weight and N derived from the soil to the amount of N derived from the atmosphere in defoliated and herbicide-sprayed swards (Table 5.1.20), showed that, as expected, fixed N in Paraggio barrel medic correlated with P fix but not with DM and nodule weight in the defoliated swards. For example, in defoliated swards the P fix was higher than in undefoliated and herbicide-sprayed swards (Table 5.1.17). N derived from the soil showed significant negative correlations with N_2 fixation in all treatments (except in the late defoliation swards). This reflects the ability of soil N to replace atmospheric N_2. Again, as expected, there was a high positive correlation between N_2 fixation and DM yield in legumes on all of the plots which demonstrates the dominant effect of DM in determining the amount of N in the swards (Table 5.1.20). Correlations involving nodule dry weight were similar to those of DM yield.

Table 5.1.20 Correlation of selected plant parameters to N_2 fixation in Paraggio barrel medic affected by defoliation and herbicide application. (Plants harvested on 4 November)

	Correlation coefficient values				
		Defoliation			
Parameters	Control	Early	Early +late	Late †	Herbicide
% P fix	0.94 ***	0.93 ***	0.79 **	0.95 *	0.88 ***
DM (kg/ha)	0.53 **	0.03	0.28	0.73	0.40*
Nodule weight (mg/plant)	0.53 *	0.17	0.14	0.03	0.40
N derived from the soil (kg/ha)	-0.87 ***	-0.86 ***	-0.64 *	-0.86	-0.77 **

* = P<0.05, ** = P<0.01, *** = P<0.001,

† The low number of data following late defoliation caused low levels of significance even though the correlation coefficients were high.

5.1.3.5 Nitrogen in roots

There were no significant interactions between sowing rate and defoliation and herbicide treatments when data for the late defoliation at 100 and 500 kg/ha sowing rates was excluded. However, sowing rate had a significant effect on % N, N yield and nitrogen derived from the soil. Defoliation and herbicide treatments also had a significant effect on N yield, P fix and fixed N from the atmosphere (Table 5.1.21).

Table 5.1.21 Summary of analyses of variance of components of N in Paraggio barrel medic roots.

Source of variation	Total N (%)	N Yield (kg/ha)	N Derived from soil (kg/ha)	P fix	N Fixed from air (kg/ha)
Sowing rate	**	*	*	NS	NS
Defoliation and herbicide	*	NS	NS	*	*
Sowing rates × Defoliation and herbicide	NS	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Over all defoliation and herbicide treatments (Table 5.1.22) the % N of roots differed significantly between swards sown at the different sowing rates. It was higher at the highest sowing rate and lowest at the lowest sowing rate.

Table 5.1.22 The effects of three sowing rates over all defoliation and herbicide treatments on the root nitrogen of Paraggio medic.

Sowing rate (kg/ha)	Total N (%)	N Yield (kg/ha)	N Derived from soil (kg/ha)	P fix (%)	N Fixed from air (kg/ha)
20	2.0	17.5	9.0	44	8.5
100	2.1	18.3	9.8	47	8.5
500	2.3	20.0	10.8	46	9.2
LSD (5%)	0.1	NS	NS	NS	NS

Changes in the amount of N derived from the atmosphere and from the soil in the roots of medic plants sampled on November 4 are shown in Table 5.1. 23. Percentage N was higher in the early+late defoliated plants in swards sown at 100 and 500 kg/ha when compared to the herbicide-sprayed plants. The amount of N derived from the soil was higher in roots of herbicide-sprayed plants than defoliated and control plant roots at the 100 kg/ha sowing rate.

In the swards sown at the low sowing rates, P fix in roots of the late-defoliated plants were higher than in control plants: this difference was not observed at the other two sowing rates. Differences, however, occurred between herbicide-sprayed, early-defoliated and control medic roots at the high sowing rates. Nitrogen fixation (kg/ha) in the herbicide-sprayed medic plant roots at the high sowing rate was dependent on P fix. Nitrogen fixed (kg/ha) was low in roots of herbicide-sprayed medic plants relative to the control at all sowing rates.

Table 5.1.23 Effects of defoliation (early on Aug.6, late on Sept.3, early+late on both Aug.6 and Sept.3) and herbicide application (2,4-DB at 2.8 L/ha sprayed on Aug.6) on the root nitrogen of Paraggio barrel medic at three sowing rates.

Treatments	N (%)	N Yield (kg/ha)	N Derived from the soil (kg/ha)	P fix	N Fixed from the atmosphere (kg/ha)
20 kg/ha sowing rate					
Control	2.0	18	8	33	10
Early defoliation	2.0	17	9	48	8
Early +late defoliation	2.1	17	9	49	8
Late defoliation	1.9	17	8	53	9
Herbicide application	1.9	18	11	38	7
LSD (5%)	NS	NS	NS	18	NS
100 kg/ha sowing rate					
Control	2.0	19	9	53	10
Early defoliation	1.9	16	8	51	8
Early +late defoliation	2.3	19	10	49	9
Late defoliation	tr	tr	tr	tr	tr
Herbicide application	2.1	20	12	37	7
LSD (5%)	0.1	NS	4	NS	NS
500 kg/ha sowing rate					
Control	2.3	22	11	51	11
Early defoliation	2.4	19	9	51	10
Early +late defoliation	2.4	20	11	46	9
Late defoliation	tr	tr	tr	tr	tr
Herbicide application	2.1	19	12	34	7
LSD (5%)	0.3	NS	NS	13	3

tr = trace amount of plant remaining after defoliation, which was not enough for sampling and was excluded from statistical analyses.

5.1.3.6 Seed yield and components

A summary of the analyses of variance for treatment effects on seed yield and seed yield components is presented in Table 5.1.24. There were no significant interactions between sowing rate and defoliation and herbicide treatments. Significant differences were found, however, between sowing rate and defoliation and herbicide treatments for seed number per m^2, seed weight and seed yield/ha.

Table 5.1.24 Summary of analyses of variance on seed yield and seed yield components of Paraggio barrel medic at three sowing rates.

Source of variation	Pods (#/m^2)	Seed:pod ratio (%)	Seeds (#/pod)	Seeds (#/m^2)	Seed weight (mg)	Seed yield (kg/ha)
Sowing rates	NS	NS	NS	*	*	*
Defoliation and herbicide	*	NS	NS	*	***	**
Sowing rates × Defoliation and herbicide	NS	NS	NS	NS	NS	NS

NS = Not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$

Over all treatments, seed yield and components of seed yield at the 500 kg/ha sowing rate were significantly lower than those at the other sowing rates (Table 5.1.25).

Table 5.1.25 Overall effects of sowing rates on seed yield and seed yield components of Paraggio barrel medic sown at three rates. (Data averaged over all defoliation and herbicide treatments).

Sowing rate (kg/ha)	Pods (#/m^2)	Seed : pod ratio (%)	Seeds (#/pod)	Seeds (#/m^2)	Seed weight (mg)	Seed yield (kg/ha)
20	4642	28	5.7	26363	4.2	1116
100	4475	27	5.5	24699	4.1	1005
500	3991	23	5.3	21099	3.9	813
LSD (5%)	565	NS	0.3	3515	0.3	159

Seed yield was correlated with pod number and seed number per m^2 in all swards. It also correlated with DM in the control swards but not for the defoliated and herbicide-sprayed swards (Table 5.1.26).

Table 5.1.26 Correlation coefficients for seed yield with components of seed yield in Paraggio barrel medic as affected by defoliation and herbicide application. (The values are means of the three sowing rates and four replications)

Component of seed yield	Correlation coefficient values with seed yield				
		Defoliation			
	Control	Early	Early+late	Late †	Herbicide
DM at 4 November	0.71 **	0.49	0.41	0.73	0.31
Pods (#/m^2)	0.82 **	0.63*	0.76 **	0.94 *	0.85 ***
Seed : pod ratio (%)	0.08	0.41	0.68 *	0.48	0.35
Seeds (#/pod)	0.46	0.79 **	0.60 *	0.48	0.84 ***
Seeds (#/m^2)	0.94 ***	0.81 **	0.86 ***	0.97 *	0.88 ***
Seed weight (mg)	0.62*	0.23	0.70 *	0.81	0.58

* = P<0.05, ** = P<0.01, *** = P<0.001,

† The low number of data following late defoliation caused low levels of significance even though the correlation coefficients were high.

Pod density (#/m^2) was highest in controls and lowest in the late-defoliated swards at the low sowing rate and was also significantly lower in the herbicide-sprayed plots than in undefoliated control plants, again at the low sowing rate. Seed density (#/m^2) and seed yield followed the same trend as pod density at the low sowing rates. At high sowing rates, maximum seed density was obtained from early-defoliated plots. These were significantly higher than those of herbicide-sprayed and control plants. Seed yield also followed the same trend as seed density in the high-sowing-rate swards. However, late defoliation significantly reduced seed yield at 20 kg/ha and produced no seed at the 100 kg/ha and 500 kg/ha sowing rates, because the plants never recovered from this defoliation (Table 5.1.27).

Table 5.1.27 Effects of defoliation and herbicide 2,4-DB application on seed yield and seed yield components of Paraggio barrel medic sown at three rates.

Treatments	Pods (#/m^2)	Seed : pod ratio (%)	Seeds (#/pod)	Seeds (#/m^2)	Seed weight (mg)	Seed yield (kg/ha)
20 kg/ha sowing rate						
Control	5764	28	5.8	33429	4.29	1439
Early defoliation	4833	28	6.0	28731	4.38	1258
Early+late defoliation	4396	29	5.6	24498	4.06	996
Late defoliation	3808	27	5.7	21721	3.91	839
Herbicide application	4410	26	5.3	23434	4.49	1049
LSD (5%)	1338	NS	NS	8966	0.29	387
100 kg/ha sowing rate						
Control	4581	28	5.6	25319	4.27	1092
Early defoliation	4876	27	5.7	27989	3.78	1070
Early+late defoliation	4307	28	5.5	23880	3.86	923
Late defoliation	†	†	†	†	†	†
Herbicide application	4137	26	5.2	21609	4.43	906
LSD (5%)	NS	NS	NS	NS	NS	170
500 kg/ha sowing rate						
Control	3730	27	5.2	19337	3.79	734
Early defoliation	4420	27	5.5	24432	4.14	1010
Early+late defoliation	3970	27	5.4	21550	3.31	709
Late defoliation	†	†	†	†	†	†
Herbicide application	3845	27	5.0	19080	4.17	801
LSD (5%)	NS	NS	NS	4305	0.50	191

† represents plots on which Paraggio medic died after late defoliation and no seed/seedling data available

5.1.3.7 Seed germination following summer rainfall

Sowing rate and the defoliation and herbicide treatments did not affect the proportion of medic seeds germinating following summer rain in Dec. 1993, Jan. 1994 and Mar. 1994. Although the emergence ranged from 67 to 136 seedlings/m^2, these seedlings all died from shortage of water.

5.1.3.8 Soil nitrogen content

No significant variations in the % N and atom $\delta^{15}N$ of the experimental area were observed during sampling in the growing season. Inorganic N appeared to increase in the soil up to 20 cm depth towards the end of the growing season. The results suggest that the soil was practically exhausted in terms of available inorganic soil nitrogen, at the first sampling in early June, by the cereal crop of the previous year: also that the medic was returning N to the soil from decomposing residues and nodules. The atom $\delta^{15}N$ in the soil was more than 6 (Table 5.1.28).

Table 5.1.28 Soil nitrogen concentration at the Waite Institute experiment site during the growing season.

Sampling	% N			Inorganic N (kg/ha)			$\delta^{15}N$ (‰)		
dates	Depth (cm)			Depth (cm)			Depth (cm)		
	0-10	10-20	20-30	0-10	10-20	20-30	0-10	10-20	20-30
3 Jul	0.11	0.07	0.05	9.5	7.6	4.7	6.2	6.6	7.1
7 Sep	0.12	0.07	0.06	12.7	16.4	5.0	6.0	6.2	6.8
11 Dec	0.12	0.07	0.07	21.2	18.9	5.6	6.1	6.7	7.3
LSD (5%)	NS	NS	NS	7.0	6.9	NS	NS	NS	NS

5.2 EXPERIMENT 6. THE EFFECTS OF MECHANICAL DEFOLIATION AND HERBICIDE 2,4-DB APPLICATION ON N_2 FIXATION, HERBAGE YIELD AND SEED PRODUCTION OF PARAGGIO BARREL MEDIC AND CLARE SUBTERRANEAN CLOVER.

(Location: field at waite Institute)

5.2.1 INTRODUCTION

Differences in dry matter production and N_2 fixation between different pasture legumes subjected to a variety of defoliation practices and/or herbicide application are common (Bolland 1991; Young *et al.* 1992). An earlier study by Black (1963) showed that prostrate cultivars were more tolerant to defoliation than tall cultivars of sub-clover. This was probably due to better survival of growing points in the prostrate cultivars than on the tall cultivars after defoliation and suggested that growth habit of the legume and its regrowth after defoliation were very important.

Although late defoliation in Experiment 5 resulted in the death of medic plants, especially at high density, the results indicated that in this case, time of defoliation *per se* was less important than the amount of leaf and the number of growing points remaining after defoliation to meet the plant's current metabolic needs in term of energy supply and regrowth. The timing of defoliation and prevailing environmental conditions have also been found to have a greater effect on sub-clover than the number of defoliations (Rossiter 1961; Collins 1978; Steiner and Grab 1986). Collins (1978) showed that defoliating sub-clover at the start of flowering increased seed yield. However Tow and Al Alkailah (1981) have reported significant reductions in seed yield in annual medics with severe defoliation before flowering.

Studies on regrowth by Gibson (1966) and Jones *et al.* (1967) indicated that the amount of nodule tissue in nodulated roots and the duration of time over which the nodules are active are very important. Chu and Robertson (1974) also suggested that after severe physiological stresses such as defoliation, roots may die or decay resulting in death or loss of nodules during regrowth.

The previous study (Experiment 5) showed greater adverse effects of herbicide application on N_2 fixation by Paraggio barrel medic than with early mechanical defoliation. To confirm this finding and determine whether it is specific to Paraggio barrel medic, a further study involving both Paraggio barrel medic and another common pasture legume, Clare subterranean clover, was undertaken at the Waite Institute. This experiment also aimed to compare the ability of these two annual legumes to recover from defoliation and herbicide application, produce herbage and seed and maintain N_2 fixation.

5.2.2 MATERIALS AND METHODS

Four main plots of subterranean clover cv. Clare were sown adjacent to the medic plots in Experiment 5. The sowing rate of both pasture legumes (100 kg/ha) was equal to the medium sowing rate used in Experiment 5. The methods used for establishment, harvesting, data collection and the treatments imposed were the same as those described in Experiment 5, except that the group C inoculant of *Rhizobium trifolii* was used for subterranean clover. Sampling of underground burr of subterranean clover involved digging burrs from the top 3 cm of the soil. These samples were sieved through a 1 mm sieve to retrieve intact burrs and any loose seed. The processing of burrs was as described for medic pods in Experiment 5.

5.2.3 RESULTS

5.2.3.1 Plant emergence

Percentage emergence on 29 June 1993 was 46% for Paraggio barrel medic and 90% for Clare subterranean clover (Table 5.2.1).

Table 5.2.1 Expected and actual emergence of Paraggio barrel medic and Clare subterranean clover when sown at 100 kg/ha.

Species	Expected plants/m^2†	Actual plants/m^2	(%) Emergence
Paraggio medic	1905	871	46
Clare sub-clover	789	708	90

† Calculated from seeds/kg of commercial seed and the germination rate.

Mean seed weights for Paraggio medic and Clare sub clover were 4.5 and 10.8 mg respectively. Hence, mean seedling density of Paraggio was higher than that for Clare, although percentage emergence was lower for Paraggio than for Clare. On the basis of sowing equal weights of pure germinable seed, more Paraggio seeds were sown resulting in a higher population of seedlings in Paraggio than Clare. Over the growing season, Clare sub clover density remained relatively constant but density of Paraggio declined by approximately 10 % by the final harvest. The most rapid decline in plant density was at the third sampling, after the late defoliation treatment had been imposed and plant regrowth had not occurred. Except at the first sampling there were no significant differences in plant number/m^2 between Paraggio and Clare (Table 5.2.2).

Table 5.2.2 Effects of species on plant density during the growing season.

	Plants (#/m^2) counted on:			
Species	29 Jun	17 Aug	14 Oct	1 Dec
Paraggio	871	833	782	761
Clare	708	719	727	719
LSD (5%)	110	NS	NS	NS

5.2.3.2. Dry matter yield

Shoots: The two pasture species differed in dry matter production throughout the growing season. Table 5.2.3 summarises the analyses of variance for data on dry matter production. At each harvest, treatment effects on dry matter yield were highly significant. But the interaction between species and defoliation and herbicide was not significant.

Table 5.2.3 Summary of the analyses of variance for shoot dry matter of Paraggio barrel medic and Clare subterranean clover.

	Harvests			
Source of variation	3 Sep	7 Oct	4 Nov	1 Dec
Species	NS	*	**	*
Defoliation and herbicide	*	**	***	***
Species × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Herbage production by Clare, when averaged across treatments, was significantly higher ($P < 0.05$) than that of Paraggio at all harvests except the first (Figure 5.2.1).

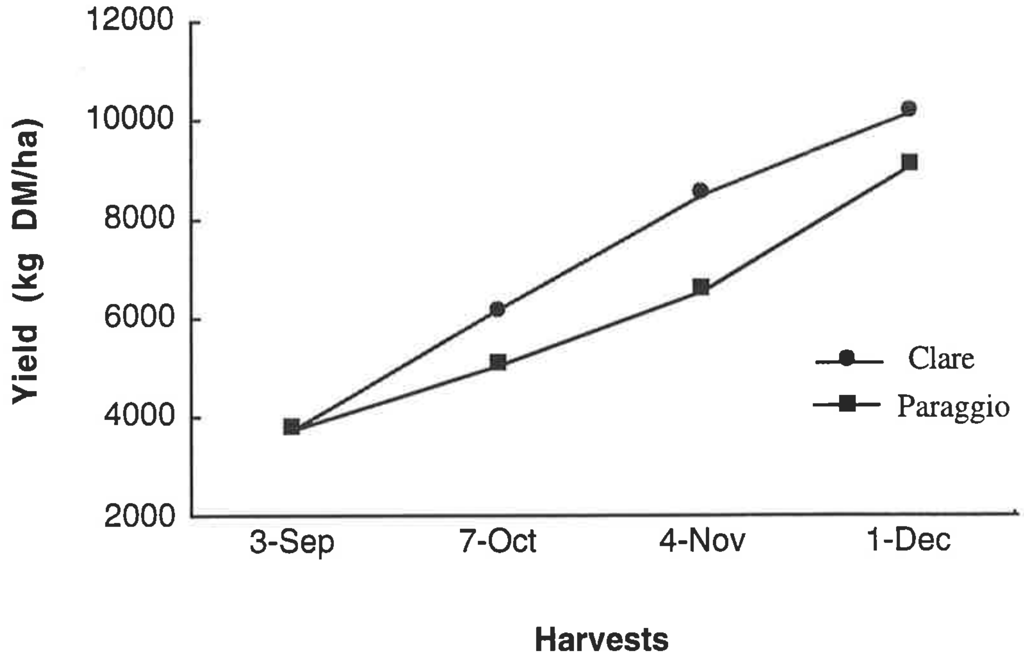


Figure 5.2.1 Mean herbage yield of Paraggio barrel medic and Clare subterranean clover throughout the growing season.

In both species, plants that had been treated with herbicide or defoliated had significantly lower dry matter yield than control plants at all harvests, except at the final harvest where dry matter production from the early-defoliated swards did not differ significantly from controls (Table 5.2.4). There were, however, two exceptions for the Paraggio barrel medic. Herbicide application significantly reduced dry matter production in Clare sub clover at the first harvest, whereas this effect was not significant for Paraggio barrel medic. With late defoliation, most of the growing points on the medic plants were removed, resulting in death of these plants. The effect of late defoliation was far less severe in Clare sub clover, than for Paraggio medic although DM yield was significantly lower than the control (Plate 5.2.1).

Table 5.2.4 Effects of defoliation and 2,4-DB herbicide application on yields of Paraggio barrel medic and Clare subterranean clover

Treatments	Yields (kg DM/ha) at harvests on:			
	Sept 3	Oct 7	Nov 4	Dec 1
Paraggio				
Control	4234	7224	9770	11184
Early defoliation	3253	6516	8657	10584
Early+late defoliation	3184	5190	6734	9460
Late defoliation	4183	tr	tr	tr
Herbicide application	4016	6259	7562	10295
LSD (5%)	617	869	898	798
Clare				
Control	4462	7578	10632	11170
Early defoliation	3260	6657	9244	10550
Early+late defoliation	3281	5715	6771	9805
Late defoliation	4226	4530	6663	9284
Herbicide application	3595	6129	9107	10208
LSD (5%)	645	606	1326	820

tr = trace amount of plant material remaining after late defoliation, which was not enough for sampling and excluded from statistical analyses.

Plate 5.2.1 **Species differences in response to the late defoliation due to differences in the proportion of leaf and stem materials.**

Symptoms of herbicide injury resulting in growth retardation and dark green colour in Clare subterranean clover (Plate 5.2.2) were obvious.

Plate 5.2.2 Effects of herbicide application on plant vigour and expression of leaf symptoms. A = Unsprayed control, B = Sprayed with 2,4-DB.

Roots: As shown in Table 5.2.5 and Figure 5.2.2 there was no interaction between species and defoliation and herbicide for any of the root parameters. However, root dry matter and depth of rooting differed significantly between the two species.

Table 5.2.5 Summary of analyses of variance on roots of Paraggio medic and Clare subterranean clover.

Source of variation	Root dry matter	Rooting depth	Nodule dry matter
Species	**	***	NS
Defoliation and herbicide	NS	NS	NS
Species × Defoliation and herbicide	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

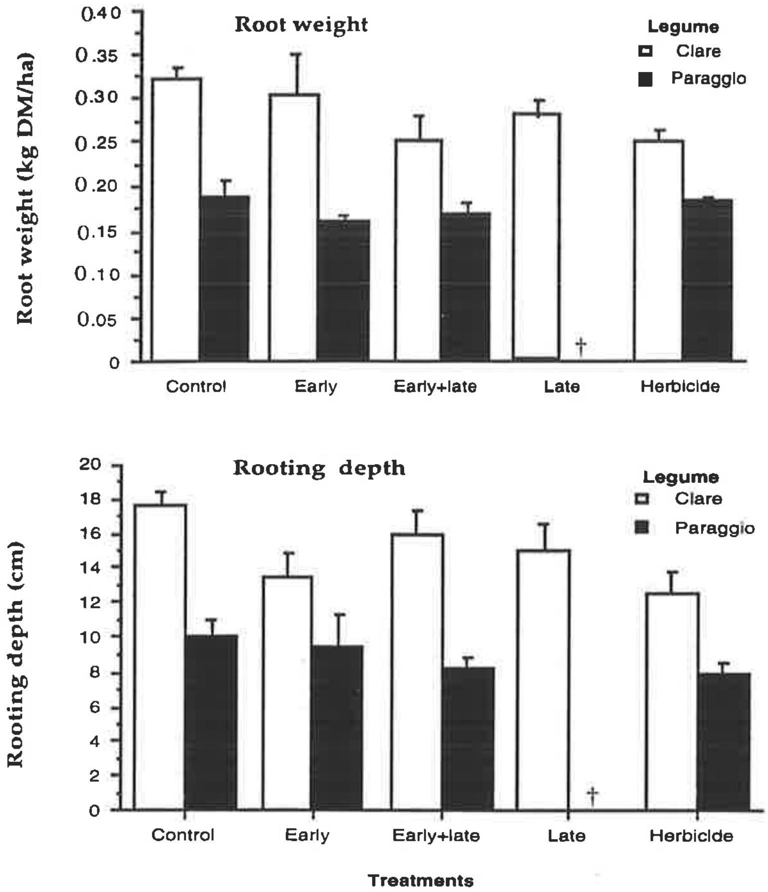


Figure 5.2.2 The effects of treatment on root weight and rooting depth of Paraggio barrel medic and Clare subterranean clover at time of harvest. (Values represent the mean of four replicates while the bars indicate S.E., † represent trace of Paraggio medic after late defoliation, which was not sampled).

Over all treatments, mean root dry weight and rooting depth of Clare were 1.6 and 1.7 times greater than for Paraggio respectively (Table 5.2.6), but nodule dry weight was not significantly different between the two species.

Table 5.2.6 Species differences in root dry matter, rooting depth and nodule dry weight of Paraggio barrel medic and Clare subterranean clover sown at 100 kg/ha.

Species	Root dry matter (g/plant)	Rooting depth (cm)	Nodule dry weight (mg/plant)
Paraggio	0.18	9	26
Clare	0.28	15	30
LSD (5%)	0.03	2	NS

The effects of defoliation and herbicide application on root properties of both species are shown in Table 5.2.7. Root dry matter production responded to defoliation and herbicide application in a similar manner in both species. Depth of rooting in Clare subterranean clover subjected to early defoliation, or sprayed with herbicide was significantly lower than that of control plants. In the Paraggio barrel medic the reduction was not significant.

Table 5.2.7 Effects of defoliation and 2,4-DB herbicide application on root data for Paraggio barrel medic and Clare subterranean clover.

Treatments	Root dry matter (g/plant)	Root depth (cm)	Nodule dry weight (mg/plant)
Paraggio			
Control	0.187	10.0	26.8
Early defoliation	0.160	9.5	28.4
Early +late defoliation	0.170	8.2	25.9
Late defoliation	tr	tr	tr
Herbicide application	0.186	8.0	24.3
LSD (5%)	NS	NS	NS
Clare			
Control	0.323	17.8	35.6
Early defoliation	0.304	13.5	27.9
Early +late defoliation	0.252	16.0	29.6
Late defoliation	0.281	15.0	25.9
Herbicide application	0.251	12.5	30.2
LSD (5%)	NS	3.6	6.6

tr = trace amounts of medic remained after late defoliation, which was not enough for sampling and excluded from statistical analyses.

5.2.3.3 Nodules

Over all treatments mean nodule weight, although 15% higher for Clare than for Paraggio, did not differ significantly between the two species (Table 5.2.6). Defoliation in Clare sub clover reduced nodule weight significantly (P< 0.05) relative to the control, but the effect was not significant in Paraggio barrel medic (Table 5.2.7).

5.2.3.4 Nitrogen in plant shoots

Percentage N (% N) and Total plant N: There was a significant Species × Defoliation and herbicide interaction for % N at the second harvest only. The species effect was also significant at the second harvest only. The effect of defoliation and herbicide treatments on % N was highly significant throughout the sampling period (Table 5.2.8).

Table 5.2.8 Summary of analyses of variance on %N in plant shoots of Paraggio barrel medic and Clare subterranean clover.

	Harvests			
Source of variation	Sept 3	Oct 7	Nov 4	Dec 1
Species	NS	**	NS	NS
Defoliation and herbicide	**	***	***	***
Species × Defoliation and herbicide	NS	***	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Over all defoliation and herbicide treatments, there were significant differences in shoot N yield between the two species on Oct 7, Nov 4 and Dec 1 (Table 5.2. 9).

Table 5.2.9 N yield of Paraggio barrel medic and Clare subterranean clover averaged over all defoliation and herbicide treatments.

	Plant N yield (kg N/ha) at harvests on:			
Species	Sept 3	Oct 7	Nov 4	Dec 1
Clare	149	174	180	211
Paraggio	150	129	139	169
LSD (5%)	NS	32	35	41

Total N in Paraggio and Clare (Table 5.2.10) generally increased with time and showed a similar pattern of changes as observed for dry matter yields. In the herbicide-sprayed plots and in both species, DM yields were lower than those of the controls and significant at the second harvest for Clare and at the third for Paraggio. Differences in total plant N between herbicide-sprayed plants and that of early+late defoliated plants at the second harvest were significant for both species.

Table 5.2.10 Effects of defoliation and 2,4-DB herbicide application on total N yield of Paraggio barrel medic and Clare subterranean clover.

	Total N (kg N/ha) at harvests on			
Treatments	Sept 3	Oct 7	Nov 4	Dec 1
Paraggio				
Control	152	161	198	214
Early defoliation	149	155	175	202
Early+late defoliation	149	185	161	218
Late defoliation	157	tr	tr	tr
Herbicide application	144	145	164	213
LSD (5%)	NS	26	23	NS
Clare				
Control	167	189	206	216
Early defoliation	141	181	181	201
Early+late defoliation	145	186	165	211
Late defoliation	161	150	160	226
Herbicide application	132	164	186	203
LSD (5%)	NS	17	30	NS

tr = trace amounts of medic remained after late defoliation, which was not enough for sampling and excluded from statistical analyses.

Proportion of fixed N (P fix): There were no significant interactions between defoliation and herbicide and species in the proportion of fixed N from the atmosphere (P fix). However, P fix (%) in the early-defoliated plants was significantly higher than that of herbicide-sprayed medic and clover plants at the Nov. 4 and Dec. 1 samplings (Tables 5.2.11 and 5.2.12).

Table 5.2.11 Summary of analyses of variance on P fix (%) of Paraggio barrel medic and Clare subterranean clover.

	Harvests			
Source of variation	Sept 3	Oct 7	Nov 4	Dec 1
Species	NS	NS	NS	NS
Defoliation and herbicide	NS	NS	*	***
Species × Defoliation and herbicide	NS	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Table 5.2.12 Effects of defoliation and 2,4-DB herbicide application on P fix (%) of Paraggio barrel medic and Clare subterranean clover.

	P fix (%) at harvests on:			
Treatments	Sept 3	Oct 7	Nov 4	Dec 1
Paraggio				
Control	67	62	51	18
Early defoliation	75	78	73	29
Early+late defoliation	76	74	65	30
Late defoliation	66	tr	tr	tr
Herbicide application	65	52	48	10
LSD (5%)	NS	NS	22	15
Clare				
Control	69	76	61	21
Early defoliation	72	66	64	26
Early+late defoliation	73	70	59	25
Late defoliation	70	70	53	37
Herbicide application	67	69	41	10
LSD (5%)	NS	NS	20	11

tr = trace amounts of medic remained after late defoliation, which was not enough for sampling and excluded from statistical analyses.

N_2 fixation: In general, there were no significant differences between the medic and clover for N_2 fixation over all defoliation and herbicide treatments except for late defoliation when the medic plants did not recover after mowing.

Changes in N_2 fixation due to defoliation and herbicide in both species are shown in Table 5.2.13. Defoliation and herbicide did not significantly affect N_2 fixation in either species compared to controls. Generally N_2 fixation was lower in herbicide-sprayed plants than defoliated plants in both species.

Table 5.2.13 Effects of defoliation and 2,4-DB herbicide application on N fixed by Paraggio medic and Clare subterranean clover.

	N fixed from the atmosphere (kg/ha) at harvests on:			
Treatments	Sept 3	Oct 7	Nov 4	Dec 1
Paraggio				
Control	101	99	100	39
Early defoliation	114	119	127	43
Early+late defoliation	113	135	105	66
Late defoliation	104	tr	tr	tr
Herbicide application	95	77	72	21
LSD (5%)	NS	36	38	34
Clare				
Control	116	142	118	44
Early defoliation	103	119	133	53
Early+late defoliation	105	132	97	53
Late defoliation	113	105	87	62
Herbicide application	89	113	76	22
LSD (5%)	NS	NS	49	27

tr = trace amount of medic remained after late defoliation, which was not enough for sampling and excluded from statistical analyses.

5.2.3.5 Nitrogen in plant roots

A significant Species × Defoliation and herbicide interaction was obtained for percentage N and N yield. Nitrogen derived from the soil was significantly different between medic and clover, while % N, P fix(%) and N fixed from the atmosphere were significantly different from the control for the defoliation and herbicide treatments (Table 5.2.14).

Table 5.2.14 Summary of the analyses of variance for the components of N in the roots of Paraggio barrel medic and Clare subterranean clover.

Source of variation	N (%)	N yield (kg/ha)	N derived from the soil (kg/ha)	P fix (%)	N fixed from the atmosphere (kg/ha)
Species	**	**	***	NS	NS
Defoliation and herbicide	*	NS	NS	*	*
Species × Defoliation and herbicide	*	*	NS	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

Figure 5.2.3 shows the effects of defoliation and herbicide on N_2 fixation in the two species. The amount of N fixed in roots was reduced in subterranean clover cv. Clare following early and late defoliation and for the herbicide treatment both relative to the control. The late-defoliated medic plots did not recover after defoliation .

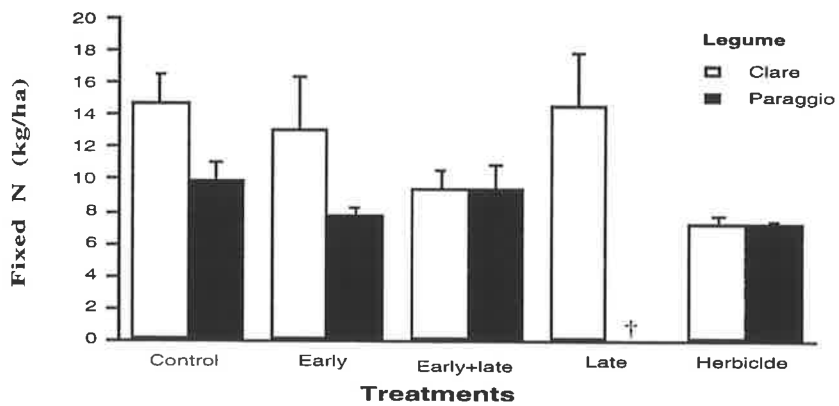


Figure 5.2.3 The effects of defoliation and herbicide on fixed N of roots in Paraggio and Clare. (Bars represent S.E. of four replicates. † represents trace amounts of Paraggio medic remaining after defoliation).

The effects of defoliation and herbicide on the components of nitrogen in the roots of Paraggio and Clare are presented in Table 5.2.15. Percentage N in roots of the early+late defoliated Paraggio plants was higher than that of the control, early-defoliated and herbicide-sprayed plants. Nitrogen derived from the soil in the herbicide-sprayed plots was also higher than that of the early-defoliated plots. There was little change in the proportion of fixed N and N derived from the atmosphere in Paraggio roots. The proportion of fixed N and the amount of fixed nitrogen from the atmosphere in roots of Clare were significantly lower in herbicide-treated plants compared with that of controls and later-defoliated plants. There was little change in the amount of N derived from the soil and % N in Clare roots.

Table 5.2.15 Effects of defoliation and 2,4-DB herbicide application on root nitrogen of Paraggio barrel medic and Clare subterranean clover.

Treatments	N (%)	N yield (kg/ha)	N derived from the soil (kg/ha)	P fix (%)	N fixed from the atmosphere (kg/ha)
Paraggio					
Control	2.0	19	9	53	10
Early defoliation	1.9	16	8	51	8
Early+late defoliation	2.3	19	10	49	9
Late defoliation	tr	tr	tr	tr	tr
Herbicide application	2.1	20	12	37	7
LSD (5%)	0.1	NS	4	NS	NS
Clare					
Control	2.4	31	16	48	15
Early defoliation	2.4	30	17	43	13
Early+late defoliation	2.4	24	15	40	9
Late defoliation	2.6	29	14	50	15
Herbicide application	2.5	25	18	29	7
LSD (5%)	NS	NS	NS	19	6

tr = trace amounts of medic remained after late defoliation, which was not enough for sampling and excluded from statistical analyses.

5.2.3.6. Seed production

Analyses of variance of seed yield and seed yield components (Table 5.2.16) showed no significant interactions between species and defoliation and herbicide treatments. The effects of defoliation and herbicide were only significant for number of seeds/pod or seeds/burr.

Table 5.2.16 Summary of analyses of variance of seed yield and seed yield components of Paraggio barrel medic and Clare subterranean clover.

Source of variation	Pods or burrs ($\#/m^2$)	Seed : pod or burr ratio (%)	Seeds (#/pod or burr)	Seeds ($\#/m^2$)	Seed weight (mg)	Seed yield (kg/ha)
Species	*	*	***	**	***	NS
Defoliation and herbicide	NS	NS	*	NS	NS	NS
Species × Defoliation and herbicide	NS	NS	NS	NS	NS	NS

NS = Not significant, * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$

Except for total seed yield which did not differ significantly between the two pasture legume species, all other seed components differed significantly between the two species. Seed weight in Clare was double that of Paraggio and was probably the major factor contributing to the differences between the two species (Table 5.2.17).

Table 5.2.17 Overall effects of defoliation and herbicide on seed yield and seed yield components of Paraggio barrel medic and Clare subterranean clover.

Species	Pods and burrs ($\#/m^2$)	Seed : pod or burr ratio (%)	Seeds (#/pod or burr)	Seeds ($\#/m^2$)	Seed weight (mg)	Seed yield (kg/ha)
Paraggio	4475	27	5.5	24699	4.1	1005
Clare	3578	35	3.1	11052	8.8	983
LSD (5%)	472	5	0.3	2638	0.5	NS

Herbicide application significantly reduced seed yield in Paraggio and Clare from 1090 to 906 kg/ha and from 1190 to 791 kg/ha respectively (Table 5.2.18). However, defoliation did not influence seed yield in either species except late defoliation of medic which caused death of the plants.

Table 5.2.18 Effects of defoliation and 2,4-DB herbicide application on seed yield and seed yield components of Paraggio barrel medic and Clare subterranean clover.

Treatments	Pods or burrs ($\#/m^2$)	Seed : pod or Seed:burr ratio (%)	Seeds (#/pod)	Seeds ($\#/m^2$)	Seed weight (mg)	Seed yield (kg/ha)
Paraggio barrel medic						
Control	4581	28	5.6	25319	4.3	1092
Early defoliation	4876	27	5.7	27989	3.8	1070
Early +late defoliation	4307	28	5.5	23880	3.9	923
Late defoliation	†	†	†	†	†	†
Herbicide application	4137	26	5.2	21609	4.4	906
LSD (5%)	NS	NS	NS	NS	NS	170
Clare sub clover						
Control	3832	39	3.2	12503	9.4	1190
Early defoliation	3554	44	3.3	11833	9.6	1134
Early +late defoliation	3497	33	3.1	10938	8.2	892
Late defoliation	3527	35	3.2	11359	8.4	956
Herbicide application	3498	27	2.6	8980	8.8	791
LSD (5%)	NS	12	0.5	NS	1.4	343

† represents plots on which Paraggio medic died after late defoliation and no seed/seedling data available

5.2.3.7 Seed germination following summer rainfall

Seed germination following summer rain and suitable cool temperatures depends on the percentage of soft seed free of dormancy . The extent of germination is measured by seedling emergence but this can never be completely accurate as many seeds may germinate but not emerge to be counted. A summary of the analyses of variance (Table 5.2.19) shows that the most important result was a significant Species × Defoliation and herbicide interaction at the first sampling.

In comparing the germination patterns of Paraggio medic and Clare sub clover following summer rains (4 December count) it should be understood that Paraggio medic would invariably have a higher percentage of hard seed (i.e. impermeable seed) than would Clare sub clover: hence the far greater germination and emergence of Clare sub clover. Furthermore, the extent of dry pasture residues, in response to defoliation and herbicide treatment, greatly influences diurnal temperature fluctuations at the soil surface, thereby leading to breakdown of hard seed of both species - hence the significant interaction.

Table 5.2.19 Summary of analyses of variance on data for seedling emergence of Paraggio barrel medic and Clare sub clover.

Source of variation	Emergence (%) when sampled on:		
	4 Dec 1993	25 Jan 1994	29 Mar 1994
Species	**	NS	NS
Defoliation and herbicide	*	NS	*
Species × Defoliation and herbicide	**	NS	NS

NS = Not significant, * = P<0.05, ** = P<0.01, *** = P<0.001

A higher proportion of seeds germinated in the early and early+late defoliated plots at the first count in Clare sub clover than for Paraggio medic, but at the second and third counts germination was higher in the herbicide-treated plots of Paraggio medic than Clare sub-clover (Figure 5.2.5). Once germinated, the soft seed (permeable seed) of Clare sub clover will be scarce hence the lower emergence counts at the second and third counts.

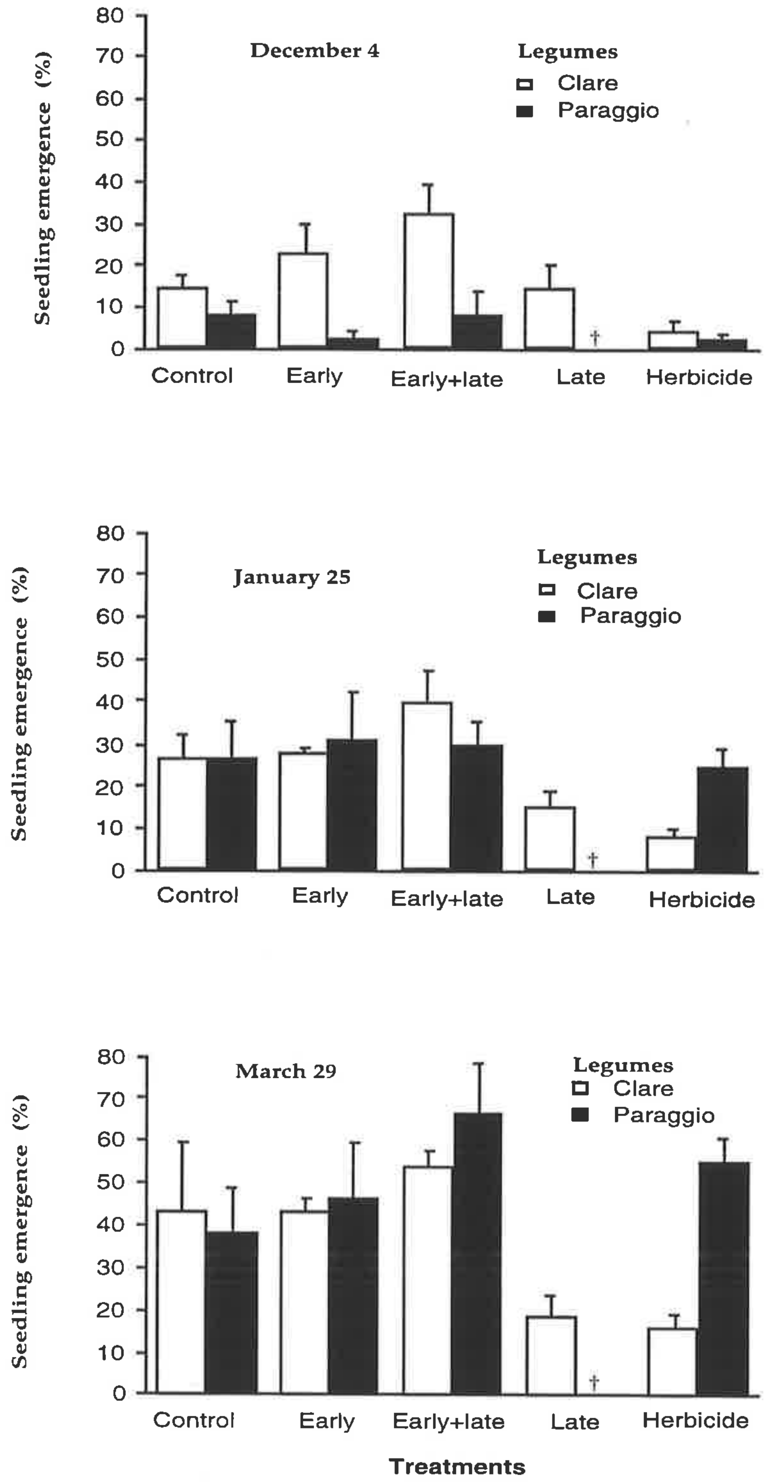


Figure 5.2.5 **The effects of treatments on seedling emergence (percentage of total seed) in the field for Paraggio medic and Clare sub clover counted on Dec.4, Jan.25 and Mar.29.** (Bars represent S.E. for four replicates. † represents plots on which Paraggio medic died after late defoliation and no seed/seedling data available).

Over all defoliation and herbicide treatments, seed germination as measured by seedling emergence, was higher in Clare than Paraggio at the first sampling: thereafter, germination was similar in the two species (Table 5.2.20).

Table 5.2.20 Overall effects of defoliation and herbicide on seed germination percentage (Measured as seedling emergence) in Paraggio barrel medic and Clare subterranean clover.

	Emergence (%) sampled on:		
Species	Dec 4, 1993	Jan 25, 1994	Mar 29, 1994
Paraggio barrel medic	5	28	52
Clare sub clover	18	23	35
LSD (5%)	8	NS	NS

Seed germination in the herbicide-sprayed Clare sub clover plots was lower than that in control plots on all sampling dates (Table 5.2.21). The effect was significant on 25 January and 29 March samplings. In contrast, germination in the early+late defoliated plots was higher than that on control plots in all sampling occasions, and was significant at the first sampling. Seed germination in defoliated/herbicide treated plots of Paraggio did not differ from the controls.

Table 5.2.21 Percentage seed germination (measured as seedling emergence) in Paraggio barrel medic and Clare sub clover on the experiment site following summer rains.

Species and treatments	Dec 4, 1993	Jan 25, 1994	Mar 29, 1994	Mean
Paraggio medic				
Control	8	26	38	24
Early defoliation	12	31	47	30
Early+late defoliation	8	29	66	34
Late defoliation	†	†	†	†
Herbicide application	2	25	55	27
LSD (5%)	NS	NS	NS	
Clare sub clover				
Control	14	26	43	28
Early defoliation	23	28	44	31
Early+late defoliation	33	40	54	42
Late defoliation	15	15	19	16
Herbicide application	4	8	16	9
LSD (5%)	15	16	23	

† represents plots on which Paraggio medic died after late defoliation and no seed/seedling data available

5.3 DISCUSSION OF BOTH EXPERIMENTS

When the effects of the herbicide 2,4-DB, defoliation and sowing rate on herbage dry matter yield, seed yield and components, nodulation and N_2 fixation in two annual pasture legumes (Paraggio barrel medic and Clare subterranean clover) were evaluated, results showed an influence of treatments on these plant attributes. In both species, control plants produced more herbage than the herbicide-sprayed plants. Growth of herbicide-sprayed plants appeared retarded with a reduction in the size of the leaflets (Plate 5.1.6). This may have caused the reduction in dry matter production. The highest shoot yields were obtained from control swards at all harvests. In Paraggio, shoot yields in controls at the final harvest (11680 kg/ha) were similar to those reported by Muyekho (1993) under field conditions at Waite Institute, while in herbicide-sprayed plots yields were reduced by approximately 10%. The results support the findings of the first experiment (Chapter 3) in which dry matter yields in Paraggio and most of the annual medics showed a reduction when the herbicide 2,4-DB was sprayed under glasshouse conditions. The herbicide also reduced rooting depth in both legumes, although the reduction was not significant in Paraggio medic. This could be ascribed to differences in root development in the two annual pasture legumes. Root dry matter and rooting depth in Clare sub-clover were approximately twice that of Paraggio barrel medic at the end of the season. The inhibition of shoot and root development by herbicide application was probably responsible for the decreased DM production in these pasture species (see discussion of Experiment 3 in Chapter 4).

Except in Paraggio medic at the highest sowing rate, seed yield was reduced significantly in all the herbicide treatments. The reduction in seed yield at all sowing rates except the highest in Paraggio may suggest that vegetative growth in the swards sown at the highest rate was more vigorous than those of the other swards, the less vigourously growing swards were therefore more disadvantaged by the herbicide application than the more vigorous-growing swards. According to Carter (1988) reducing excessive vegetative growth by spraying low rates of herbicide prior to flowering is one of the management practices used by annual medic seed producers in South Australia to maximise seed yield. The reduction in seed yield of both species at the low sowing rates when the herbicide was applied may probably be a consequence of reduced assimilate supply since herbicide also reduced shoot dry matter production. Reduction in seed yield in subterranean clover following the application of herbicide in spring in Victoria has been reported by Schroder and Stapleton (1992): in one of their experiments the average seed yield was 511 kg/ha in control plots and 211, 343 and 318 kg/ha respectively where dicamba, 2,4-DB amine or glyphosate were applied.

In the present Waite Institute experiment, the partitioning of assimilate between the seed and pod wall (seed:pod ratio) differed between the plant species. In Clare sub clover, the seed:pod ratio was reduced by the herbicide application, while in Paraggio there was a small, non-significant reduction in seed:pod ratio (Table 5.2.18). This could be attributed to seed number per pod in the plants and suggests that, when canopy photosynthesis is limiting and seeds per pod are not reduced (as shown in Paraggio, Table 5.2.18). There is possibly remobilisation of assimilates from the pod wall for seed growth (Summerfield *et al.* 1976).

The adverse effects of the herbicide 2,4-DB on nodulation was only observed in swards sown at low rates (Table 5.1.9). At the high sowing rate, nodule dry weight was significantly lower than in plants grown at the low sowing rate. The lower nodule dry weight in swards at the higher sowing rate was associated with greater plant numbers in these sites, but resulted in a reduction of the phytotoxicity of the herbicide on individual plants. Therefore under field conditions it is difficult to predict whether reductions in nodulation are a direct result of herbicide application or an indirect effect of plant number. However, the reduction in nodule dry weight by the herbicide supports the results of the glasshouse experiment, which showed a significant reduction in nodule number in Paraggio medic plants.

Over all herbicide-treated plants, changes in N_2 fixation did not correlate with the changes observed in nodulation and dry matter yield (Table 5.1.20). This supports the view expressed in Chapter 3, that the effect of herbicides on legume-*Rhizobium* symbiosis may be mediated through effects on the host plants. Alternatively, after treatment with the herbicide the activity of the remaining nodules might increase to compensate for the reduced number of nodules.

The results obtained from N_2 fixation calculated from the field experiment were in contrast to those obtained from the glasshouse experiment (Experiment 1) in which the application of 2,4-DB caused lower rates of AR activity compared to controls. The difference may be due to the different conditions under which the two experiments were conducted. This experiment was carried out under field conditions and N_2 fixation was measured by the ^{15}N natural abundance technique which estimated N_2 fixation integrated over time from emergence to harvest. The results observed under glasshouse (controlled) conditions were measured by the acetylene reduction assay, over a short time period and may not therefore reflect the true N_2 fixation activity of the legumes over the entire growing season.

Growth rate of all defoliated swards (except late defoliation in 100 kg/ha and 500 kg/ha sowing rates) (Figure 5.1.3) was rapid and equalled the dry matter production of control plants at the end of the growing season, suggesting that although defoliation removed most of the foliage, the new (regrowth) foliage produced changed their overall physiological age and increased their growth rate. This may explain the similarity in dry matter yields between controls and early-defoliated swards at the final harvest (Table 5.1.6). However, the cumulative dry matter yields in defoliated swards at the first two harvests (Table 5.1.4) were significantly lower. Basic limitations to regrowth determined by species caused the different defoliation practices to have different effects on the two legumes tested. The fact that the Clare sub-clover has a more prostrate habit than the Paraggio medic meant that it lost a lower percentage of photosynthetic tissue than the medic when defoliated and therefore recovered more rapidly.

The effects of defoliation on plant attributes was influenced by the timing and frequency of defoliation and the sowing rate and consequent density. The highest shoot recovery occurred in swards sown at 20 kg/ha. These swards had a more rapid regrowth after each defoliation treatment, because at the time of defoliation, higher density swards were taller and therefore had a lower proportion of leaf and stem material remaining after defoliation than those sown at 20 kg/ha. The poorest recovery was in the swards sown at both 100 kg/ha and 500 kg/ha after the single late defoliation treatment. The recovery patterns for swards sown at the low and high sowing rates differed when defoliated at the late stage of growth. The single late defoliation led to death of plants in the swards sown at 100 kg/ha and 500 kg/ha whereas regrowth occurred rapidly in the early+late defoliated treatments in the same swards. This was probably due to apical retardation of most of the main stem, primary and secondary branches after the first defoliation. Many of the apices of these branches were probably still below cutting height at the time of the second defoliation which enabled the early-defoliated sward to survive the second defoliation. The recovery pattern for late-defoliated swards in subterranean clover, however, differed from that of Paraggio, presumably because of the differences in the proportion of leaf and stem material remaining after defoliation. The results support the assertion that as the intensity of defoliation increases herbage yield decreases (Donald 1951). In both species, cummulative dry matter production in twice-defoliated (early+late defoliated) swards was lower than that of the controls.

Early defoliation of swards sown at higher sowing rates resulted in higher seed yields than in the other defoliation systems (Table 5.1.27). The advantage of early defoliation at higher sowing rates over the other defoliation treatments was probably due to decreasing plant heights and build up of strong stems. This may reduce lodging in the swards and led to a rapid development of new leaves. Early defoliation (before flowering) therefore did not affect pod number per m^2 and seed yield. Reduction in seed yield with late defoliations in the experiment was through a reduction in the number of pods per m^2.

The immediate post-defoliation rates of N_2 fixation were not estimated because several workers have reported that N_2 fixation after defoliation is not constant and generally N_2 fixation after defoliation increases exponentially after a few days. Rate of recovery of N_2 fixation after defoliation was presumably related to the increasing activity of the remaining photosynthetic surface and supports the hypothesis that symbiotic N_2 fixation in nodulated legumes appears to depend on products of photosynthesis (Butler *et al.* 1959). Kim *et al.* (1993) showed that shoot removal from *Medicago sativa* grown hydroponically caused a severe decline in N_2 fixation within 6 days after cutting and remained low until day 10 of regrowth. After day 11, the rate of recovery increased, with N_2 fixation rate on day 24 exceeding the initial value. Moustafa *et al.* (1969) also found that defoliation caused a reduction in the rate of N_2 fixation (decreased rate of acetylene reduction activity) during the first day after defoliation. However, the rate began to rise after the sixth day. This rise was probably associated with the production of new leaves. Table 5.1.23 shows that the amount of N_2 fixed by nodulated roots, estimated from the sample harvested on November 1 before the final harvest, was not significantly altered in defoliated plants. This shows that the effect of defoliation on N_2 fixation in nodulated plant roots was short lived and may have no long-term important effects on the potential of the defoliated plants to fix nitrogen (with exception of late-defoliation of the medic swards sown at 100 and 500 kg/ha, which did not recover after defoliation).

Nitrogen fixation correlated with dry matter yields in control swards but not in defoliated swards (Table 5.1.20). This was probably because the amount of fixed N did not decrease following defoliation despite the decreased level of dry matter production by the defoliated swards. These results suggest that the level of fixed N in defoliated swards was controlled more by P fix rather than by plant dry matter yield. The persistence of total fixed N following defoliation (Figure 5.1.8) was associated with an increase in P fix in the defoliated swards (Table 5.1.17), which probably resulted from the growth of younger, more-active plant material. Therefore, N_2 fixation in defoliated swards did not correlate with dry matter yields. This finding is in agreement with the study by Høgh-Jensen and Schjoerring (1994) where they used two cutting intensities and 6 cuts per season on a mixture of white clover (*Trifolium repens* L.), red clover (*Trifolium paratens* L.) and ryegrass (*Lolium perenne L.*) and estimated N_2 fixation by the ^{15}N dilution method. They found that frequent cutting had no deleterious effect on the N_2 fixation processes. On the basis of the present results (Figure 5.2.3) Clare subterranean clover appears to have higher N_2 fixation rates than Paraggio barrel medic when subjected to defoliation.

Sowing rate was the most important factor interacting with defoliation and herbicide application. Shoot DM for Paraggio medic was low at the low sowing rate and high at the high sowing rate early in the season. At the end of the season, the reverse occured as shoot DM was high at the low sowing rate and low at the high sowing rate. This was probably due to death of some of the plants at the high sowing rates at the end of the season (Table

5.1.5). Root dry matter yields and depth per plant at the final harvest also tend to decrease with increasing sowing rates (Table 5.1.8). This could be attributed to poorer growth at the higher sowing rates than at the lower sowing rates due to an excessive number of plants at the higher sowing rates. These results are supported by earlier studies of Donald (1951, 1954) which indicated that final dry matter yield in annual pasture legumes was constant in moderate-density to high-density swards.

The decline in seed yields and seed yield components with increases in sowing rates (Table 5.1.25) shows the importance of the sward being able to supply adequate photosynthate during pod-development to ensure high seed production. This is in agreement with the work of Cocks (1988) and Muyekho (1993). The seed yield in Paraggio (1439 kg/ha) in the control swards at 20 kg/ha was higher than that obtained by Adem (1977) (500 kg/ha), and 1300 kg/ha reported by Muyekho (1993) at the Waite Institute. Analysis of the seed yield components (Table 5.1.27) indicated that at low sowing rates, seed yield in Paraggio medic was determined by the number of pod per m^2. This finding is in agreement with the work of Cocks (1988; 1990).

The results of the Waite Institute studies also show an effect of sowing rate on N_2 fixation during the growing season. While at the lower sowing rate N_2 fixation varied between 74 kg N/ha on 3 Sept and 78 kgN/ha on 1 Dec (despite an increase in shoot dry weight from 2789 kg/ha to 10346 kg/ha over the period), a large decrease in N_2 fixation which ranged from 132 kg N/ha on 3 Sept to 44 kgN/ha on 1 Dec was observed at the higher sowing rate (despite an increase in shoot dry weight from 4563 kg/ha to 7690 kg/ha over the period) (Table 5.1.19). This shows that the pattern of N_2 fixation over a growing season may be influenced considerably by sowing rate (plant density) and may not be directly related to dry matter accumulation. The change in the proportion of N due to fixation (P fix) in relation to total N during plant growth at different sowing rates (densities) may be responsible for this. While P fix was reduced by about 40% in the low sowing rate throughout the growing season, a reduction of 70% was obtained at the higher sowing rate. This could be related to more competition for light or essential nutrients at the higher sowing rates than at low sowing rates.

At the first harvest, atmospheric N was the predominant source of N for all of the swards sown at the different sowing rates (Figure 5.1.9). Thereafter fixed N from the atmosphere decreased and soil-N uptake tended to increase in all of the swards until the end of the growing season. The increase in percentage soil-N taken up during the latter part of the growing season was probably due to decreased atmospheric-N fixation by the plants. Decrease of atmospheric-N in the plants was due to the decrease of P fix in the plant during the latter part of the growing season when the plants were mature. This suggests that Paraggio medic was unable to continue fixing N_2 from the atmosphere in fairly constant amounts up to the end of the growing season probably due to lack of soil moisture. Nitrogen fixation also tended to decrease earlier in swards sown at higher sowing rates than at lower rates, because soil moisture will obviously run out sooner at the higher sowing rates. An earlier reduction in P fix was observed in swards at the higher sowing rates (Table 5.1.16 and 5.1.19). Soil derived N in the plants increased appreciably at the end of the season. This could be because legume N_2 fixation has declined due to a lack of soil moisture and the legume has taken up soil-available N which has apparently accumulated at the end the season. Jensen (1987) and Zapata *et el.* (1987a,b) have reported that legumes continued to accumulate soil-N until the end of the growing season.

Comparison of seed yield in the early-defoliated and the herbicide treatments showed that Paraggio was more sensitive to herbicide application than to early defoliation (Table 5.1.27). Also a marked difference in seed yield between early-defoliated swards and herbicide treated swards of subterranean clover in the same experiment confirms the greater sensitivity of the clover to herbicide application. A reduction in seed:burr ratio by herbicide application was also observed in subterranean clover (Table 5.2.18). The similarity in seeds per pod between the treated and untreated swards of Paraggio sown at all rates suggest that seeds per pod in annual medics is relatively stable under management practices such as defoliation and herbicide application. The results are in agreement with studies by Muyekho (1993) at the Waite Institute.

Comparison of N_2 fixation in the early defoliation and herbicide treatments showed that herbicides and defoliation *per se* do not appear to significantly affect N_2 fixation in the annual legumes. Although some reduction in N_2 fixation in the plants sprayed with herbicide and a slight increase in N_2 fixation in early defoliated plants were observed, the differences were not significant when compared to the controls, but significant when herbicide treated plants are compared with early defoliated swards. This observation suggests that where pasture weeds can be removed by defoliation, early defoliation of legume pastures for weed control (as an alternative to herbicide application) can improve N_2 fixation processes in the pastures.

In the field, the $\delta^{15}N$ enrichment of the soil ranged from 6.0 to 7.3 irrespective of soil depth, which validated the use of the natural abundance of ^{15}N method to estimate N_2 fixation in the field. The soil depth may be less important because most of the inorganic N is found in the upper 20 cm of soil. The $\delta^{15}N$ enrichment of the reference plant averaged 4.85, reflecting $\delta^{15}N$ enrichment of the extracted soil N (Table 5.1.28)

CONCLUSIONS

The present Waite Institute experiments show that the effects of defoliation and herbicide application on Paraggio barrel medic and Clare sub clover varied with sowing rate (and consequent density) and differed between the two pasture legumes. Timing and frequency of defoliation was an important factor influencing the success of defoliation strategies.

Defoliation at the early stages of plant growth has the potential to increase seed yield in high sowing rate (high density) swards through the initiation of younger foliage and increased tillering.

The study suggests that cutting at a height of 5 cm late in the growing season could cause the death of Paraggio medic in plants in high-density swards because of almost total removal of photosynthetic tissue and growing points.

There was a slightly-adverse effects of the herbicide application and a slight-positive effects of early defoliation on N_2 fixation. Therefore, N_2 fixation in herbicide-sprayed plants was significantly lower when compared to early-defoliated plants. This suggests that despite resultant stress from defoliation, production of young plant material and presence of new nodules after early defoliation leads to improve N_2 fixation in the defoliated legumes.

The study shows that in situations where pasture weeds can be removed by defoliation, pasture production and particularly N_2 fixation can be improved by early defoliation of the sward. At the same time, seed production in Paraggio barrel medic is also increased by early defoliation.

Finally these two experiments provide a factual basis for well-established practices of continuous grazing of annual medic and subterranean clover pastures, early hard grazing at high stocking densities for weed control and cutting hay 'early and high' to ensure clover and medic plant survival and regrowth of high-quality herbage for livestock feed and /or seed production.

CHAPTER 6

6.1 GENERAL DISCUSSION

This chapter first discusses factors affecting the methods of measurement of N_2 fixation used in this thesis and then discusses the results of the experiments in terms of the effects of herbicides and defoliation on growth and N_2 fixation of the pasture legumes studied. Some comparisons between the effects of these two treatments are made and the possibility of using defoliation as an alternative to herbicide for weed control is discussed.

One of the major objectives of this thesis research was to contribute to knowledge of the effects of herbicide on N_2 fixation by pasture legumes. This could lead to a reduction in herbicide use by farmers.

6.1.1 ESTIMATES OF N_2 FIXATION

Considerable attention has been given to the ^{15}N natural abundance method of estimating N_2 fixation in the legumes used in the experiments. During this study (field experiments at Kapunda and the Waite Institute) the $\delta^{15}N$ abundance in the available soil N was measured 6 (‰) or greater. This indicated that the ^{15}N natural abundance method was the appropriate one to use (Domenach and Corman 1984; Ledgard and Peoples 1988 and Peoples *et al.* 1989). This is because the reference plant, which receives all of its N from the soil, absorbs N which approximates the isotopic composition of the soil N abserbed by the legume. Therefore, lower enrichment reduces the accuracy of the method since the dilution in ^{15}N by atmospheric N decrease ^{15}N abundance proportional to the amount of N fixed.

In this method, the choice of an appropriate non-N_2 fixing reference plant is known to be particularly important (Witty 1983b). Annual ryegrass was used as the reference plant in the field experiments at Waite Institute for estimating N_2 fixation in Paraggio medic and Clare sub clover by the ^{15}N natural abundance method . The ryegrass matured at a similar time to Paraggio and Clare in the swards, and its pattern of growth was quite similar to that of the Paraggio and Clare. Therefore, it was considered a suitable reference plant in thse experiments. This is in agreement with the findings of Ledgard *et al.* (1985c) that ryegrass was a suitable reference for subterranean clover in their experiments, although it has been found that the suitability of a reference plant may change under different environmental and climatic conditions (Fried *et al.* 1983). In general, it is recommended that the legume and reference plant should be well matched in their pattern of growth with time and also depth of rooting; although, as reported by Peoples *et al.* (1991), this is probably less important with the natural ^{15}N abundance method, which has been used in this study, than with the ^{15}N enrichment method.

During this study, the amount of N derived from the atmosphere in medic and clover was estimated (Experiment 4, Chapter 4) by using *B* value, reflecting fractionation of ^{15}N and ^{14}N in the fixation process (Turner and Bergersen 1983). The *B* values of Paraggio medic also Clare and Mt Barker sub clovers were assessed in N-free culture solution in a naturally-lit glasshouse at the Waite Institute. Unkovich *et al.* (1994) used different *Rhizobium* strains and reported a marked effect of *Rhizobium* strain on *B* values in sub clover cultivars. Therefore, the medic and sub clover were inoculated with the specific recommended *Rhizobium* strains for those species which was the same strain used in the field experiments.

Care was taken to avoid common problems in use of the acetylene reduction assay for estimating comparative relative rates of N_2 fixation in the range of annual pasture legumes used in the first experiment. Sand was used as the growth medium, because it holds a relatively constant volume of water and / or nutrient solution during plant growth and assay. Un-damaged intact plants were used in their pots and covered with sheets of transparent plastic to enable light to enter during the assay. The assay was conducted at the growth temperature to prevent any influence of a change in temperature.

For the sake of accuracy it was decided to estimate N_2 fixation on shoots and roots of legumes and reference plants separately, although the sample preparation and analytical costs were doubled. This was because it has previously been reported that estimating N_2 fixation in one plant part may not represent N_2 fixed in the whole plant (Ladd 1981; Phillips *et al.* 1983). In the current studies that the ^{15}N enrichments in the legume and reference plants differed between plant shoots and roots. This is consistent with the findings of Fried *et al.*

(1983), Butler and Ladd (1985b) and Butler (1987) where the distribution of ^{15}N in various plant parts has been reported to be different. However, it is suggested that if ^{15}N is uniform in different plant parts (Danso *et al.* 1993), there is no need to estimate different organs separately. Even when N_2 fixation was high, Ledgard *et al.* (1985b) found similar N_2 fixed in the shoot and in the whole plant of the clover, although there were differences in ^{15}N in different parts of the clover and the reference plant.

In this thesis study, the sampling and estimating of ^{15}N in roots was repeated several times, as high variation was observed between the root samples. This variation can probably be attributed to several factors. The first relates to the sampling method used, for example selecting small volumes for root sampling (soil cores 14.7 cm diameter). The second is sample measurement; root yields were small so any inaccuracies in the method of measurement would result in relatively high percentage errors. The third factor relates to the effects of environmental influences, rather than the effects of the treatments; the distribution of roots is very dependent on the physical properties of the soil and/or variation in soil structure.

In the two field experiments at the Waite Institute, the root sampling was done before medic pod development, because it was assumed that N_2-fixing activity in annual medics decreased from this stage until the end of the season. This is in agreement with the findings of many researchers that nitrogenase activity and N_2 fixation in annual legumes peak at the flowering stage, and decrease sharply at pod development (Peoples and Dalling 1988). The data from the experiments confirmed this assumption and showed that N_2 fixation in low density swards peaked at early pod fill. The results also showed that in swards at higher densities the peak amount of fixed N_2 occurred one month earlier than in the low density swards, and thereafter decreased progressively during pod fill. Sutton (1983) has suggested that this is probably because of the competition between pods and nodules for carbohydrate supply but also it may well be due to earlier depletion of soil water under the higher-density swards.

6.1.2 EFFECTS OF HERBICIDES

Glasshouse and field experiments conducted from 1992 to 1995 provided evidence of a range of tolerance to herbicide by different species and cultivars of annual pasture legumes. Efficient herbicide use was related to rate and time of the herbicide application and the use of a wetting agent.

A comparison of the results of these experiments indicated both similarities and differences in the response of the legumes to herbicide application and also showed that the inhibition of legume production and N_2 fixation by the herbicides was dependent on herbicide concentration and time of application. In most experiments, the herbicides were applied at different stages of plant growth to evaluate the efficiency of the herbicides applied at different times, but in the two final field experiments at the Waite Institute the herbicide was applied when the swards were well established (c.two months after sowing). Up to this stage of plant growth, swards may be more sensitive to other types of environmental stress such as water shortage, high temperature and soil acidity, which influence the onset of N_2 fixation (Gibson 1971, 1976, 1977). Nevertheless, the effect of the herbicide sprayed on the legume foliage at the fully-established stage of the swards, was associated with changes in leaf morphology and scorching of leaves, which could be clearly seen (Plate 5.2.2). This may have been a response to reduction of photosynthesis and subsequently reduced photosynthate supply. The long-term effect of reduced photosynthate supply is probably reduced nodulation.

These observations agree with the study by Cardina *et al.* (1986), who found that the reduction of nodule activity in a legume plant (crown vetch) by a herbicide (atrazine) was probably due to a decrease in photosynthesis. They also concluded that the effects of herbicide application on nodulation and N_2 fixation are probably due to phytotoxicity rather than interference with rhizobial growth or direct effects on nitrogenase activity. DeFelipe *et al.* (1987), using lindex and simazine also reported detrimental effects of herbicide on the nodulation process in *Lupinus albus* L. The simazine altered the nodule cells by affecting vesicle formation, degenerated the bacteria, and reduced the number of bacteroids for N_2

fixation. They concluded that these herbicides had different effects on nitrogen fixation and photosynthetic mechanisms. However, the effect of a herbicide on a symbiotic system may appear at two stages (Martensson and Nilsson 1989): first, the root hair infection may be inhibited resulting in the formation of insufficient nodules and second, the formed nodule activity may decrease, probably because the formation of amino acids is stopped.

The data from the first experiment show a significant Herbicide × Cultivar interaction (Table 3.1.2). This same interaction was also reported in studies by Mulholland *et al.* (1989) and Evans *et al.* (1989). They recommended selecting new cultivars for tolerance to the herbicide used and investigating the effects of new herbicides on recommended cultivars. However, differential effects of herbicides on dry matter production and N_2 fixation found in this study, suggest the possibility of selecting legumes for herbicide tolerance.

It is recognised that the stage of growth at which herbicides are applied may markedly affect the plant's response. There was good evidence that, of the 10 annual medics tested in the first experiment, Harbinger medic showed most tolerance to the herbicide 2,4-DB when sprayed at the first trifoliate leaf stage, but this herbicide significantly reduced dry matter yield and AR activity of the Harbinger when sprayed at the fifth trifoliate leaf stage. This agrees with the finding of Young *et al.* (1992) who showed a significant reduction in DM yield in Harbinger treated by 2,4-DB sprayed 53 days after sowing, while Mulholland *et al.* (1989) found that Harbinger was quite tolerant of post-emergence spraying of 2,4-DB.

In the glasshouse experiment, 2,4-DB reduced DM yield of Paraggio in most of the treated swards (Figure 3.1.1) and the level of nitrogenase activity was also lower than that of the control plants 24 days after treatment. In the field experiment, although DM yield of Paraggio was significantly reduced by 2,4-DB, N_2 fixation was not changed significantly by the herbicide application. In fact the effect of 2,4-DB on N_2 fixation of Paraggio in the glasshouse experiment was different from that in the field experiment. Environmental factors, presumably both physical and biological, may have influenced the herbicide activity in the field.

In Experiment 2, simazine at 2.0 L/ha markedly reduced nodule numbers in Clare sub clover sown in a pure culture (Figure 3.2.2). However, in the grass/legume pasture in Experiment 3, the application of simazine+paraquat and simazine+fluazifop-P at recommended rates increased the nodule number in the sub clover relative to the unsprayed grassy control swards. This was probably because of reduction of competition from grass weeds in the mixed sward, suggesting that in the grassy pasture the legume and nodules were suffering more from competition from grass weeds than from damage by the herbicide. This is in agreement with the finding of Butler and Ladd (1985b) who found that in a mixed sward when there was a 10% increase in ryegrass dry matter, there was a 10% decrease in the amount of N fixed by Harbinger medic.

In most cases the herbicide treatment changed the legume nodule colour from pink to a darker colour. A similar change in nodule colour from pink to green caused by physiological stress was reported by Virtanen *et al.* (1947) to be due to degradation of leghaemoglobin within the nodule.

The addition of wetting agent was found to be an important factor increasing the efficiency of the simazine+paraquat herbicide combination which reduced grass weeds in the pasture and increased the N_2 fixation by the clover. This confirms the findings of Cudney *et al.* (1993) that the addition of wetting agents to the amine form of 2,4-DB improved weed control, and did not result in alfalfa injury. Bovey *et al.* (1988) also concluded that the addition of wetting agents to 2,4-D and clopyralid (3,6-dichloro-2-pyridinecarboxylic) can improve the efficiency of these herbicides.

The findings of this thesis research indicate that management stress, such as herbicide application, which decreases herbage production during the growth period could be expected to reduce seed yield. Yet, commercial medic seed producers in South Australia use both heavy grazing by sheep and herbicide applications at low rates to control vegetative growth before flowering with the aim of increasing seed yield. However, the current findings indicate that the recommended rates of herbicide for weed control should not be used for controlling excess vegetative growth in the legume sward.

6.1.3 EFFECTS OF DEFOLIATION

In this thesis research program the effects of defoliation depended on sward density and demonstrated the importance of plant species and defoliation patterns in determining the relative herbage production and N_2 fixation. Within dense swards, the lower parts of the control (undefoliated) plants became subject to deeper shading as further canopy development occurred. In those circumstances, the plants lost lower leaves under the canopy; further effects were yellowing, wilting and even death of whole plants. However, in defoliated swards sown at the same density this effect was deferred. There was rapid regrowth and canopy development following defoliation: however, the subsequent rate of growth varied with plant species and time and frequency of defoliation.

In Experiments in the field at the Waite Institute, late defoliation of Paraggio in the two higher-density swards resulted in death of the plants, while late defoliation did not cause the death of plants in the swards sown at low rates, or in the previously-defoliated, higher-density swards. Swards in the two latter treatments also recovered from the defoliation. These results indicate that the time of defoliation is not itself the critical issue; more important is the ability of the residual leaf materials following defoliation to meet the plant's current metabolic needs in terms of energy and potential regrowth. Late defoliation of the medium density (100 kg/ha sowing rate) and high density (500 kg/ha sowing rate) Paraggio medic resulted in negligible residual leaf area and growing points hence the death of the plants. The response to late defoliation differed for Clare sub clover in Experiment 6; where productive regrowth occurred successfully after late defoliation. This marked difference between medic and sub clover results from the fact that, even at the medium and high density, the Clare sub clover, with a more-prostrate growth habit, had significant residual leaf area and population of growing points following late defoliation.

Seed yields in the Paraggio medic and Clare sub clover used in the two field experiments at the Waite Institute were reduced as a result of early+late, and late defoliation, but not for early defoliation treatments. This suggests that the reduction in seed yields following defoliation was mainly due to the presence of immature seeds, because the seeds on defoliated plots did not have time to mature normally before the onset of dry weather. However, beneficial effects of early defoliation will vary depending on the climatic conditions throughout the growing season and also plant densities. In our experimental situation, it appears that early defoliation (before flowering) had little effect on dry matter production and seed yields of swards resulting from lower sowing rates, but increased dry matter production and seed yields at higher sowing rates.

There was evidence that defoliation resulted in reduced nodule dry weight in Paraggio medic and Clare sub clover in both the defoliation experiments. A significant reduction in the nodule dry weight was observed in low density swards after early+late defoliation (Table 5.1.9). This was consistent with results of research by Ryle *et al.* (1985a), which showed increased senescence of large nodules in severely-defoliated white clover when compared with undefoliated plants. They concluded that when defoliation stress is not severe and plants are still relatively young, the roots and nodules will remain unscathed. However, it must be noted that the growth form of white clover is completely different from that of medics or sub clover. Chu and Robertson (1974) described three ways in which a reduction in nodule number and or nodule weight may occur after severe physiological stress such as defoliation practices: (i) roots, and therefore nodules, may die after severe treatment. (ii) roots may decay and slough off, which causes loss of nodules and (iii) individual nodules may be lost, leaving empty 'hulls' on roots. Ryle *et al.* (1985a) also showed that defoliation stress particularly decreases the ability of nodules to react to increased supply of photosynthate. In Paraggio, no reduction in nodule weight and N_2 fixation was observed after early defoliation when the plants were sampled at the beginning of the pod stage. This indicated that in N_2-fixing legumes, defoliation has no long-term effects on nodule production during regrowth. The result is consistent with the finding on white clover nodules (Chu and Robertson 1974) that the N_2 fixation function of individual nodules was temporarily reduced by defoliation, but that bacterial activity tended to increase during the recovery period.

The amount of fixed nitrogen in defoliated plants generally tended to increase approximately one month after defoliation, relative to the undefoliated plants, although the increased level was not significant during sampling periods. The results from this study and those observed by other workers suggest that defoliation may have immediate adverse effects on the N_2 fixation processes, but that these effects decline during recovery from defoliation. This is in agreement with the findings of Ryle *et al.* (1985a) that the recovery of N_2 fixation after defoliation was a response to the regeneration of the photosynthetic system. Ta *et al.* (1990) also reported interruption of the current photosynthate supply caused by shoot defoliation. Therefore, defoliation, by stimulating new growth and thereby increased activity of the photosynthetic surface, could be expected to lead to increased N_2 fixation after recovery. In the Waite Institute field experiments, the defoliated plants contained more P fix than undefoliated control plants at most of the harvests (Table 5.1.23). The significant finding of these two experiments was the higher level of P fix and no reduction of nitrogen fixed by the most-defoliated legumes relative to the undefoliated (control) legumes. Similarly Høgh-Jensen and Schjoerring (1994) reported that frequent cutting had no deleterious effect on N_2 fixation processes in white clover plants.

Defoliation appeared to increase the percentage nitrogen content of Paraggio medic and Clare sub clover shoots in Experiments 5 and 6, although there were no consistent differences in percent nitrogen content of roots between defoliated and undefoliated plants. With some contrasts with these results, Ryle *et al.* (1985b) concluded that, in N_2-fixing legumes, defoliation has no long-term effects on the organic nitrogen content of the herbage produced during regrowth. However, the severity of defoliation, its timing and other stress factors could be expected to affect the results of defoliation.

6.1.4 COMPARISON OF EFFECTS OF HERBICIDE WITH DEFOLIATION

The purpose of this study was to investigate the effect of herbicide on herbage production and N_2 fixation of annual pasture legumes and to find ways to overcome the problems. The results from these experiments and those of other workers suggest that in most cases both herbage production and N_2 fixation of annual pasture legumes are affected detrimentally by herbicides. In Experiments 5 and 6 at the Waite Institute, where mechanical defoliation was used as an alternative to herbicide application in field conditions, the overall finding indicated that neither herbicide application nor defoliation practices had significantly changed N_2 fixation activity in the treated plants, when compared to the controls. Although there was evidence of only a slight adverse effect from the herbicide, and a slight positive effect from defoliation on most occasions, the cumulative effects of herbicide and defoliation combined to give marked and significant differences between the two treatments.

However, it must be stated that choice between using herbicide or defoliation will depend on the species of weed or weeds in the legume pasture also the percentage botanical composition. In many on-farm situations, either defoliation by topping with a mower in late winter or spring or a herbicide may be used to control weeds and increase the percentage of legume in pasture.

Finally, on most broadscale farms the well-established method of controlling weeds by intensive grazing by sheep (Carter 1990a) is unlikely to change . However, it is important to have a range of options for weed control and to know the biological and economic implications of using herbicides, mechanical defoliation and grazing for weed control in legume pastures having regard to ensuring sustainable farming systems.

6.2 GENERAL CONCLUSIONS AND SUGGESTED FURTHER WORK

The research detailed in this thesis suggests that :

1. Efficient herbicide use in legume pastures could be promoted by using more effective formulations, sufficient but not excessive rates, wetting agents, and by adjusting timing of application according to plant species and stage of growth.

2. When it is possible to control weeds in pasture by mechanical defoliation rather than herbicide application, nitrogen fixation by pasture legumes can be increased.

3. The findings of the research described in this thesis and information available from other studies are limited, and there is need for further experimentation in a wide range of environmental conditions and seasons to confirm the importance of pasture management strategies for annual legume pastures. This will need to involve ongoing research on:

- Pasture management, to enhance the understanding of the complexity of the Management $\times$ Plant species $\times$ N_2 fixation interactions in pastures for different climates and soils.
- The integration of non-chemical techniques for weed control with limited use of more efficient herbicides in pastures leading to more effective grass control and increased N acquisition through nitrogen fixation.
- Effects of herbicides on new pasture species and cultivars to assess their susceptibility.
- Defoliation management strategies to optimise pasture production and N_2 fixation.
- Additional alternatives to chemical weed control in pastures to reduce herbicide use and avoid contamination of soil and water resources and ensure that there is no contamination of animal products used for human consumption.

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