The Effects of Acetolactate Synthase (ALS) Inhibiting Herbicides on the Growth, Yield, Nodulation and Nitrogen Fixation of Selected Legumes

Annette Anderson

B.Sc. (Hons). James Cook University

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Agricultural and Natural Resource Sciences at Adelaide University.

Department of Agronomy and Farming Systems

Roseworthy Campus

Adelaide University

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Abstract

Acetolactate synthase (ALS) inhibiting herbicides are widely used in the cereal growing regions of South Australia and include sulfonylureas, sulfonamides and imidazolinones. Sulfonylureas, such as chlorsulfuron, are used in cereal crops of southern Australia to control broadleaf weeds. Sulfonylurea residues have been found to inhibit the growth of some legume crops and pastures in seasons following application. Sulfonamides (e.g. flumetsulam) and imidazolinones (e.g. imazethapyr) are recommended for weed control in legume crops and pastures. Yellowing and stunting of growth of legumes has been observed in the field following applications of some sulfonamides and imidazolinones. It is possible that these herbicides are impacting on symbiotic nitrogen fixation of legume crops or pastures. This study investigated the effects of ALS-inhibiting herbicides on the growth and grain/seed production of chickpea and medic and the symbiotic nitrogen fixation of chickpeas.

The presence of triasulfuron or chlorsulfuron residues reduced the shoot biomass of *Medicago rugosa,* by 31 – 60%. Flumetsulam alone had no effect on *M. rugosa* shoot biomass. Seed yield of *M. rugosa* was not affected by any of the herbicides.

A second field trial investigated the effects of four application rates of chlorsulfuron in combination with 'in-crop' applications of flumetsulam or imazethapyr on *Cicer arietinum* (chickpea) growth, yield and nitrogen fixation. Chlorsulfuron at 0.75, 1.5 and 3 g active ingredient (ai) ha^{-1} (the recommended application rate is 15 g ai ha^{-1}) reduced shoot biomass by 22%, 36% and 49% respectively. Imazethapyr (29g ai ha^{-1}) reduced chickpea shoot biomass by 52%, whilst flumetsulam had no significant effect on shoot biomass. Chickpea yield was affected by an interaction between

chlorsulfuron and imazethapyr. Chlorsulfuron (at 1.5 and 3.0 g ai ha^{-1}) reduced nitrogen fixation (kg N fixed ha^{-1}) by 40% and 57% respectively. Imazethapyr reduced nitrogen fixation by 52%. The results for nitrogen fixation reflected those of shoot biomass, so the results were converted to amount of nitrogen fixed per unit of shoot biomass. Only imazethapyr reduced nitrogen fixation after this conversion.

Investigations into the effects of chlorsulfuron and imazethapyr on nodulation were conducted in a pot experiment. Imazethapyr or chlorsulfuron were present or absent, (i) during the growth of rhizobia prior to inoculation, (ii) pre-germination of chickpea seeds, and (iii) in the soil in which the plants were grown. The presence of either chlorsulfuron or imazethapyr in the soil reduced the nodulation of chickpea plants. Pre-exposing rhizobia to chlorsulfuron or imazethapyr reduced the number of nodules formed on chickpea plants by 51% and 35% respectively, in the absence of herbicides in the soil or at seed germination.

In vitro studies investigating the effects of chlorsulfuron, imazethapyr, and flumetsulam, at double the recommended application rate, on the growth of chickpea *Rhizobium* (CC1192) in yeast mannitol broth revealed that there were no significant effects on bacterial growth, at either pH 7.0 or pH 8.0. Similar results were observed when rhizobial cultures were grown in a defined media, without amino acids, in the presence of chlorsulfuron.

An experiment using ^{14}C labelled chlorsulfuron was conducted to determine if pre-exposed rhizobial cells were delivering herbicides to the point of root infection and nodule formation. Approximately 1% of the herbicide present in the rhizobial growth medium remained on the cells after rinsing with ¼ strength Ringer's solution. This equated to approximately 2.04×10^{-18} g ai rhizobial $cell^{-1}$, and this concentration is

unlikely to injure the plant. It appears from the outcome of these studies, that ALS-inhibiting herbicides have a negative impact on the formation of symbiotic nitrogen fixing root nodules, even when only the rhizobial inoculant is exposed briefly to the herbicide, possibly by interfering with the nodulation infection process.

The impacts of ALS-inhibiting herbicides reported in this study have implications for farming systems, in terms of reduced biomass, yield and soil nitrogen balance. A reduction in nitrogen fixation potentially leads to soil fertility problems due to reduced nitrogen input to the soil. Preliminary estimates of the potential costs to farmers in terms of yield and the requirement to apply additional nitrogen fertiliser, were up to \$388 ha^{-1}. Future studies are required to investigate the effects of these herbicides on the symbiotic root nodule infection and formation process in more detail.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Annette Anderson

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CHAPTER 1

GENERAL INTRODUCTION

1.1 LEGUMES, NITROGEN FIXATION AND ALS-INHIBITING HERBICIDES

Farming systems are no longer aiming simply for higher productivity, but are also striving for sustainability (Peoples *et al.*, 1995b). The use of legumes in crop rotations has benefits including (i) improvement of the soil structure, (ii) increased nutrient availability especially when legume residues are incorporated into the soil, (iii) breaking disease and cereal pest cycles, and (iv) increased soil microbial activity following the addition of legume residues (Peoples *et al.*, 1992).

It is necessary for farming systems to replenish lost nutrients in order to maintain both sustainability and productivity (Peoples *et al.*, 1995b). Legumes, via biological nitrogen fixation, provide an alternative nitrogen source to fertiliser nitrogen application (Schwenke *et al.*, 1998; Peoples *et al.*, 1995b). Biological nitrogen fixation improves the nitrogen economy of soils, although including legumes in cropping systems does not always make large net contributions of nitrogen to the soils in which they grow (Unkovich *et al.*, 1997; Peoples *et al.*, 1995b). However, the nitrogen balance following a legume–cereal rotation will be higher than for a cereal–cereal rotation in the same soil (Peoples *et al.*, 1995b). Biological nitrogen fixation by legumes can be managed to overcome problems such as: soil acidity inhibiting rhizobia; management of soil nitrate levels to minimise leaching; and achievement of nitrate levels below those that inhibit early nodulation (Unkovich *et al.*, 1997).

If weeds are not controlled, they reduce crop yields, hinder harvest operations and contaminate produce (Powles *et al.*, 1996). Weeds can be controlled by a variety of methods including: (i) crop and pasture rotations, (ii) introduction of grazing animals, (iii) cultivation, and (iv) in recent years, predominantly through the use of herbicides (Powles *et al.*, 1996). Australian farmers spent more than $300 million on herbicides for weed control in pastures and crops in 1991 (Lemerle *et al.*, 1996). However, residual levels of some herbicides have been found to inhibit nodulation (Eberbach and Douglas, 1989; Martensson and Nilsson, 1989) and nitrogen fixation (Koopman *et al.*, 1995).

The acetolactate synthase (ALS) inhibiting herbicides are one group of herbicides widely used throughout Australia and include the sulfonylureas, imidazolinones and sulfonamides. The sulfonylureas are used for controlling broad leaf weeds and some grasses in cereal crops, while the imidazolinones and sulfonamides are recommended for weed control in some legume crops and pastures (Chambers, 1995). The persistence of sulfonylureas applied to cereal crops, has been reported to inhibit subsequent legume crops and pastures under alkaline soil conditions (see Table 2.6), due to insufficient or slow degradation. Stunting of growth and yellowing of leaves has been observed in field pea crops and medic pastures following applications of flumetsulam and imazethapyr (Chambers, 1995; DowElanco herbicide label; Cyanamid herbicide label). These symptoms of growth inhibition and yellowing of leaves may have consequences for the legume-*Rhizobium* symbiosis and hence the nitrogen economy of the soil.

Herbicides may affect the legume-*Rhizobium* symbiosis in a number of ways including: (i) direct effects on the host plant, (ii) reduction of rhizobial survival or growth, (iii) inhibition of the nodulation process, or (iv) influences on nitrogen fixation

(Eberbach, 1993). This thesis details a series of experiments investigating the effects of sulfonylurea residues and in-crop usage of flumetsulam or imazethapyr on the growth and seed yield of a pasture legume (*Medicago rugosa*), and the growth, grain yield and nitrogen fixation of a grain legume, chickpea (*Cicer arietinum*). In addition, the possible mechanisms responsible for the observed effects on nitrogen fixation are determined. The possible mechanisms include an examination of the effects of the herbicides on nodulation and the growth of *Rhizobium*.

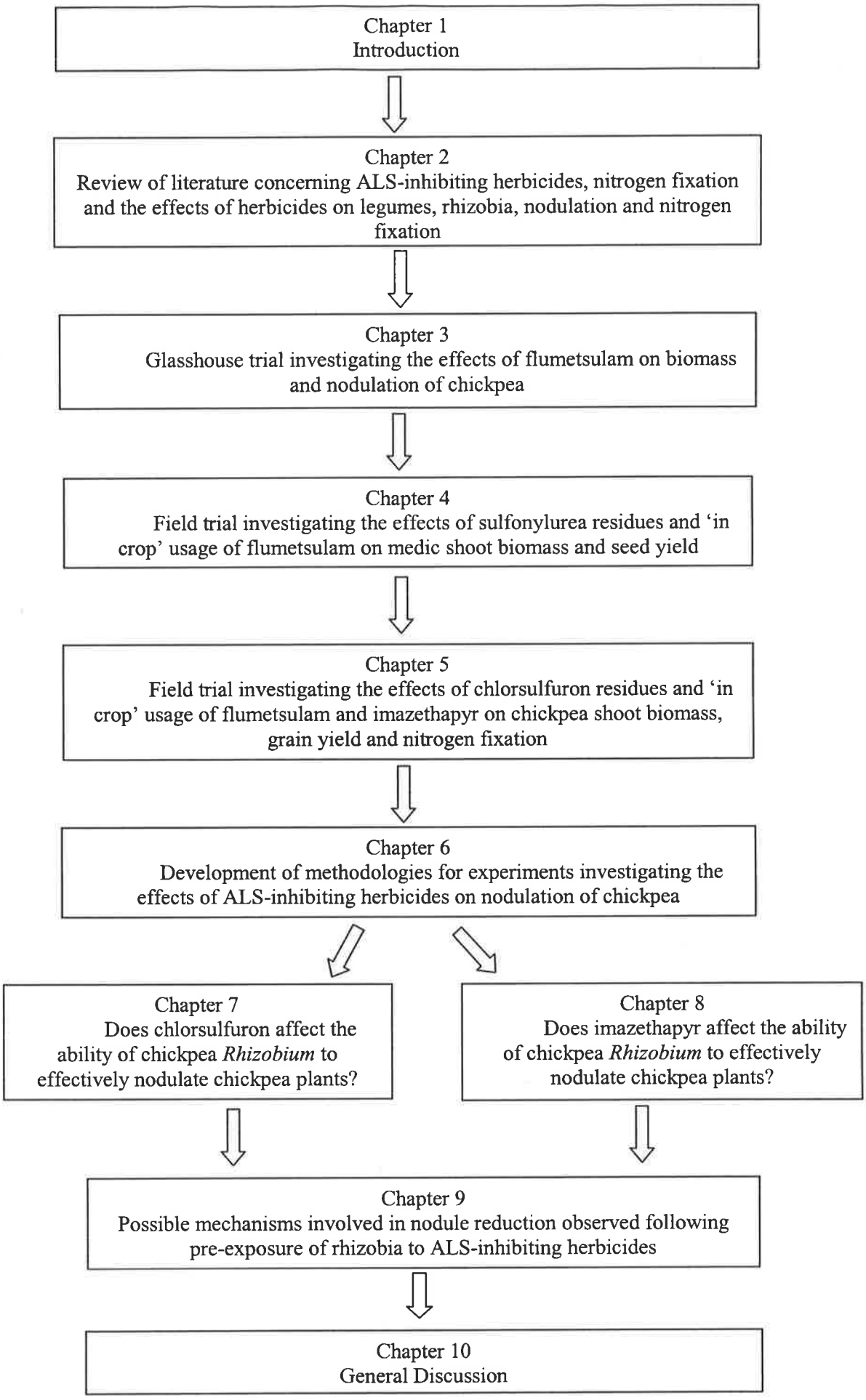
1.2 AIMS AND STRUCTURE OF THE THESIS

1.2.1 Aim of this thesis

The main aim of this thesis is to determine the effects of sulfonylurea residues and in-crop usage of selected ALS-inhibiting herbicides on the growth, yield, nodulation and nitrogen fixation of legumes under alkaline conditions.

1.2.2 Structure of thesis

The structure of the thesis is presented in Figure 1.1.


Chapter 1
Introduction
Chapter 2
Review of literature concerning ALS-inhibiting herbicides, nitrogen fixation and the effects of herbicides on legumes, rhizobia, nodulation and nitrogen fixation
Chapter 3
Glasshouse trial investigating the effects of flumetsulam on biomass and nodulation of chickpea
Chapter 4
Field trial investigating the effects of sulfonylurea residues and 'in crop' usage of flumetsulam on medic shoot biomass and seed yield
Chapter 5
Field trial investigating the effects of chlorsulfuron residues and 'in crop' usage of flumetsulam and imazethapyr on chickpea shoot biomass, grain yield and nitrogen fixation
Chapter 6
Development of methodologies for experiments investigating the effects of ALS-inhibiting herbicides on nodulation of chickpea
Chapter 7
Does chlorsulfuron affect the ability of chickpea *Rhizobium* to effectively nodulate chickpea plants?
Chapter 8
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CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

The control of weeds is important for maximising profitability of agricultural systems. If left uncontrolled, competition from weeds can decrease crop yields and weed seeds can contaminate the produce. There are a variety of methods used to control weeds including crop and pasture rotations, grazing, cultivation and herbicides (Powles *et al.*, 1996). In recent years, the use of herbicides has become widespread and is often the main method of controlling weeds due to their relative ease of use, affordability and effectiveness. There are fifteen groups of herbicides available for farm use (Table 2.1; Powles *et al.*, 1996). Group B herbicides inhibit acetolactate synthase activity in plants and belong to the biosynthesis pathway inhibitors of Moorman (1994).

Herbicides make it possible to grow crops with minimum tillage (Blacklow & Pheloung, 1992; Pratley and Rowell, 1987), which is important for reducing soil compaction and erosion (Brady, 1990). However, some groups of herbicides may create problems such as resistance and persistence.

Resistance occurs as a result of a weed population developing genetic tolerance to a herbicide that would normally be lethal to most individuals of that species. Resistance is inherited and is therefore passed onto successive generations of weeds, causing problems for farmers (Powles *et al.*, 1996). In recent years there has been a rapid worldwide increase in reported cases of herbicide resistance (Powles *et al.*, 1996).

Persistence occurs when a herbicide applied in one season fails to degrade completely and remains in the soil in sufficient quantities to injure subsequent crops, thus injuring more than the original intended target. This problem has been observed with the acetolactate synthase (ALS) inhibiting herbicides and has injured subsequent legume crops and pastures in South Australia. The problem of persistence of ALS-inhibiting herbicides and its subsequent effects on legume growth, and the capacity to interact with *Rhizobium* and fix atmospheric nitrogen forms the focus of this review.

Table 2.1: Classification of herbicides according to their mode of action (Powles *et al.*, 1996).

Group	Principal mode of action	Chemical families
A	Inhibitors of acetyl CoA carboxylase (ACCase)	aryloxyphenoxypropanoates cyclohexanediones
B	Inhibitors of acetolactate synthase (ALS)	sulfonylureas, imidazolinones, triazolopyrimidine sulfonamides
C	Inhibitors of photosynthesis at photosystem II	triazines, triazinones, phenylureas, nitriles, benzothiadiazoles, acetamides, uracils, pyridazinones, phenyl-pyridazines
D	Inhibitors of tubulin formation	dinitroanilines, pyridazines
E	Inhibitors of mitosis	carbamates, thiocarbamates, organophosphates
F	Inhibitors of carotenoid biosynthesis	nicotinanilides, triazoles, pyridazinones, isoxazolidinones,
G	Inhibitors of protoporphyrinogen oxidase	diphenyl ethers, oxadiazoles, N-phenylphthalimides
H	Inhibitors of plastoquinone biosynthesis	triketones
I	Disrupters of plant cell growth (hormone mimics)	benzoic acids, phenoxys, pyridine carboxylic acids
J	Inhibitors of cell wall synthesis	benzamides, dichlobenil
K	Herbicides with diverse sites of action	aminopropanoates, benzofurans, chloroacetamides, nitriles, phenylcarbamates, phthalamates, quinoline-carboxylic acids
L	Disruptors of photosynthesis at photosystem I	bipyridyls
M	Inhibitors of EPSP-synthase	glyphosate
N	Inhibitors of glutamine synthetase	glufosinate
O	Uncouplers of energy transfer	organoarsenicals

2.2 ACETOLACTATE SYNTHASE INHIBITING HERBICIDES

2.2.1 Introduction

The ALS-inhibiting herbicides are a relatively new group of herbicides with the first sulfonylurea herbicide discovered in 1975 by Dr George Levitt of Du Pont (Beyer *et al.*, 1987). They include the sulfonylureas, imidazolinones, and the triazolpyrimidine sulfonamides (which will be referred to as sulfonamides throughout this thesis). Examples of the major groups of ALS-inhibiting herbicides used within Australia are presented in Table 2.2. This literature review will focus on the sulfonylureas as they are the most widely used family of ALS-inhibiting herbicides.

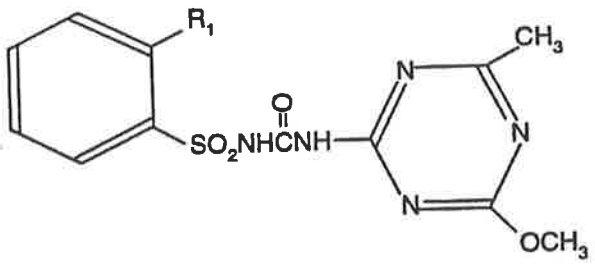
Table 2.2: Major groups of acetolactate synthase (ALS) inhibiting herbicides used in Australia.

Family	Trade name	Active ingredient	Use
sulfonylureas	Glean®, Ally®, Logran®	chlorsulfuron, metsulfuron-methyl, triasulfuron	Cereals
imidazolinones	Spinnaker®	imazethapyr, imazaquin, imazapyr	Legumes
Triazolopyrimidine sulfonamides	Broadstrike®	Flumetsulam	Legumes

Sulfonylureas are widely used by cereal growers to control broad leaf weeds (e.g. Ball mustard (*Neslia paniculata*) and soursob (*Oxalis pes-caprae*)), and some grasses (e.g. annual ryegrass (*Lolium rigidum*)) (Chambers, 1995; Blair and Martin, 1988). They are characterised by low application rates (recommended rates vary from 4 - 40 g ha^{-1} for chlorsulfuron whereas alachlor and atrazine have application rates of 500 and 2000 g ha^{-1} (Beyer *et al.*, 1988)), long term efficacy (e.g. chlorsulfuron has a half life of 32 weeks at pH 7.5 in non sterile soil (Beyer *et al.*, 1988)), good crop selectivity

and low mammalian toxicity (oral LD_{50} for sulfonylureas in rats is greater than 4000mg kg^{-1} (Beyer *et al.*, 1987)). Sulfonylureas have been adopted by many sectors of the agricultural industry (Ferris *et al.*, 1992; Brown, 1990; Beyer *et al.*, 1988; Blair and Martin, 1988). The structure and properties of three common sulfonylurea herbicides are given in Table 2.3 (Bos *et al.*, 1995). Sulfonylureas are composed of three sections: an aryl group, heterocycle portion and a sulfonylurea bridge that links the aryl and heterocyclic groups (Devine *et al.*, 1993). The structure can be seen in Table 2.3.

Table 2.3: Structures and some properties of three commonly used sulfonylurea herbicides (taken from Bos *et al.*, 1995).



Common name	R_1	Solubility in water (mg/L)	Vapour Pressure (10^{-15} Pa)	pK_a
chlorsulfuron	Cl	0.3 x 10^3 (pH 5, 25°C) 28 x 10^3 (pH 7, 25°C)	3,000 (25°C)	3.6
metsulfuron-methyl	CO_2CH_3	1.1 x 10^3 (pH 5, 20°C) 9.5 x 10^3 (pH 7, 20°C)	300 (extrapolated to 25°C)	3.3
triasulfuron	OCH_2CH_2Cl	1.5 x 10^3 (pH 7, 20°C)	0.1 (20°C)	4.5

2.2.2 Mode of action of ALS-inhibiting herbicides

The ALS-inhibiting herbicides are absorbed by both the roots and shoots of plants (Ferris *et al.,* 1992; Blair and Martin, 1988) and are transported via the xylem and phloem (Ferris *et al.,* 1992). Seed germination is usually not affected, but seedling emergence is greatly inhibited in the presence of sulfonylureas (Blair and Martin, 1988). Symptoms of sulfonylurea damage include chlorosis, terminal bud death, vein discolouration, necrosis and inhibition of growth (Brown, 1990; Ray, 1982a).

Early studies into the mode of action of sulfonylureas found that growth of sensitive plant species was severely inhibited by chlorsulfuron. In an attempt to determine whether this growth inhibition was due to inhibition of cell division or cell expansion, Ray (1982a) performed a series of bioassays using plant hormones. These bioassays found that chlorsulfuron at levels up to 10 mg kg^{-1} had no effect on indoleacetic acid-induced elongations of pea stems, cytokinin-induced cell expansion of cucumber cotyledons or gibberelic acid-induced elongation of lettuce hypocotyls (Ray, 1982a). However, cell division was inhibited in corn root seedlings at 0.01 mg kg^{-1} (Ray, 1982a). Further investigations into the mode of action found no direct inhibitory effects on DNA synthesis (Ray, 1982b).

Ray (1984) noted that the addition of the amino acids valine and isoleucine alleviated growth inhibition from chlorsulfuron in pea (*Pisum sativum*) and a similar mode of action in bean roots was noted by Klingaman and Peeper (1989). The imidazolinone herbicide AC243 997 reduced valine and leucine levels in corn (Anderson and Hibberd, 1985). Similar results were found with other sulfonylurea herbicides including nicosulfuron and rimsulfuron (Mekki and Leroux, 1994). Further studies investigating the mode of action of these herbicides found growth inhibition by

sulfometuron methyl of *Salmonella typhimurium* in the presence of valine and this inhibition was reversed in the presence of isoleucine (LaRossa and Schloss, 1984).

These studies showed that sulfonylurea herbicides act by inhibiting acetolactate synthase (also known as acetohydroxy acid synthase). Acetolactate synthase is responsible for catalysing the first step of the biosynthesis of valine, leucine and isoleucine (Hershey *et al.*, 1999; Mekki and Leroux, 1994; Brown, 1990; Anderson and Hibberd, 1985; LaRossa and Schloss 1984; Ray 1984). Valine and leucine are synthesised from pyruvate, while isoleucine is derived from α-ketobutyrate (Mousdale and Coggins, 1991). The inhibition of acetolactate synthase leads to the subsequent rapid cessation of plant cell division and growth (Brown, 1990).

Rost *et al.* (1990) have suggested that the inhibition of branched chain amino acids alone does not account for the reduction in cell division and growth resulting from treatment with imidazolinone herbicides. Two doses of Arsenal (the imidazolinone herbicide, imazapyr), 200 uM (causes inhibition of cell cycle) and 2 uM (causes partial inhibition of cell cycle) were found to reduce the branched chain amino acid pool by less than 50%, suggesting that the pool reduction itself was not the cell cycle inhibition step (Rost *et al.*, 1990). Inhibition of acetolactate synthase lead to a buildup of α-ketobutyrate (as a result of inhibiting isoleucine synthesis) that may be toxic at high concentrations (Devine *et al.*, 1993; Mousdale & Coggins, 1991).

Crop species (e.g. wheat) tolerant to these acetolactate synthase herbicides, appear to detoxify the herbicide by rapid cellular metabolism before it reaches the site of action (i.e. ALS) (Christopher, *et al.*, 1992; Brown, 1990; Hageman and Behrens, 1984; Sweetser *et al.*, 1982). In cereals, chlorsulfuron undergoes hydroxylation on the phenyl ring followed by conjugation with glucose (Sweetser *et al.*, 1982). In

broadleaves, the herbicide is hydroxylated on the methyl group of the heterocycle followed by conjugation with a sugar (Beyer *et al.*, 1988).

2.3 DEGRADATION AND PERSISTENCE OF ALS-INHIBITING HERBICIDES IN THE SOIL

Herbicides may be lost from the soil either by physical removal of the molecule or by degradation. Processes of physical removal include volatilisation at the surface, leaching, sorption and plant uptake (Fryer and Makepeace, 1977). Volatilisation, in which herbicide molecules leave the soil surface in the vapour phase and enter the atmosphere, has been shown to be of little consequence to sulfonylureas (Beyer *et al.*, 1987). Adsorption on soil colloids can also affect the activity of the herbicides in the soil by stopping the herbicides from reaching their target site and by effectively 'deactivating' the herbicide (Leake, 1991; Fryer and Makepeace, 1977). The primary particles in soils include both inorganic and organic particles. The inorganic particles are differentiated based on their size and mineralogy (Fitzpatrick, 1986). Adsorption generally increases with increases in the surface area of soil inorganic particles (primarily determined by the clay content) and with soil organic matter content (Leake, 1991). Soil adsorption of chlorsulfuron was directly related to soil organic matter (Walker *et al.*, 1989).

Degradation processes include photolysis, chemical breakdown and microbial decomposition (Fryer and Makepeace, 1977). Sulfonylurea degradation is dominated by the processes of chemical hydrolysis and microbial breakdown (Beyer *et al.*, 1988; Beyer *et al.*, 1987; Joshi *et al.*, 1985). Chemical hydrolysis predominates in acidic soils, with microbial breakdown the principal mode of degradation in alkaline soils

(Beyer *et al.*, 1987). Soil pH, soil moisture, temperature, organic matter and soil type (Moyer *et al.*, 1989) influence degradation and persistence of sulfonylurea herbicides. It is possible that the influence of soil type on herbicidal effects may be masked by the pH of the soil. It is likely that there is an interaction between pH, soil moisture, temperature, organic matter and soil type or any combination of these to bring about phytotoxic effects from the herbicides. Table 2.4 gives details of the influence of these processes on chemical degradation. Persistence of these herbicides, due to insufficient or slow degradation, can lead to problems with subsequent crops and pastures and will be discussed later in this review.

Table 2.4: Factors affecting the persistence of ALS-inhibiting herbicides in soils.

Environmental factors	Mechanism	Examples	References
pH	Sulfonylureas are weak acids (pK_a 3.3-5.2). Neutral form that is particularly susceptible to hydrolysis dominates at pH levels below the pKa value.		Bos *et al.*, 1995; Beyer *et al.*, 1987; Shea, 1986
	Sulfonylurea breakdown increases with decreasing pH.	Chlorsulfuron breakdown 15 times greater at pH 5.9 than at 8.0.	Joshi *et al.*, 1985; Beyer *et al.*, 1987
		½ life of chlorsulfuron increased from 1.5 weeks at pH 5.6 to more than 9 weeks at pH 7.5.	Fredrickson & Shea, 1986
		½ lives of Chlorsulfuron & triasulfuron ranged from 12 – 28 days at pH 5.8- 6.5 in W.A.	Blacklow &Pheloung, 1992
	Adsorption of chlorsulfuron appears to increase as pH decreases.		Shea, 1986; Mersie and Foy, 1985; Thirunarayanan *et al.*, 1985
	pH has a great effect on the dissipation of these herbicides and has been the basis for the recommended re-cropping intervals appearing on product labels.		Borggaard & Streibig, 1988 Bos *et al.*, 1995
Soil Moisture	Warm, moist soil conditions that promote microbial activity also promote breakdown of sulfonylurea herbicides.		Beyer *et al.*, 1987
	Major route of chlorsulfuron degradation is through hydrolysis that increases with increasing soil moisture.		Thirunarayanan *et al.*, 1985
	Sulfonylurea persistence increases with decreasing moisture levels and is generally attributed to the soil drying over longer periods, thus reducing rates of hydrolysis and microbial activity.	45-64% of chlorsulfuron remained 3 months after application in a dry soil, compared to 3-4% in moist soil.	Walker & Robinson, 1996
		Increasing soil moisture from 25-50% of field capacity increased degradation rate of chlorsulfuron by 46%.	Thirunarayanan *et al.*, 1985
	Increased soil water reduced chlorsulfuron degradation in a loam soil due to anaerobic microsites developing in the soil.		Anderson, 1985

Table 2.4: Factors affecting the persistence of ALS-inhibiting herbicides in soils.

Environmental factors	Mechanism	Examples	References
Soil Moisture cont..	High soil moisture following chlorsulfuron application increased the phytotoxicity to the weeds green foxtail *(Setaria vividis)* and kochia (*Kochia scoparia*) when compared to high soil moisture before application.		Nalewaja & Woznica, 1985
	Sulfonylureas are highly mobile in alkaline soils and are readily leached under high rainfall conditions.		Pederson, 1996
Temperature	Chemical hydrolysis and microbial degradation increase with increasing temperature.		James *et al.*, 1995; Atlas & Bartha, 1987; Beyer *et al.*, 1987
	Degradation of ALS herbicides increases with increasing temperatures.	½ lives of primisulfuron methyl and metsulfuron methyl decreased by 50 and 75% respectively when temperature was increased from 10 – 30°C.	James *et al.*, 1995
		Chlorsulfuron had a ½ life of 229 days at 10°C and 62.5 days at 40°C.	Thirunarayanan *et al.*, 1985
	Inverse relationship between sulfonylurea phytotoxicity and temperature.	Fewer peas emerged after treatment with chlorsulfuron at 5°C than at 30°C.	Joshi *et al.*, 1985
		Chlorsulfuron phytotoxicity to the weeds kochia and green foxtail was lower at 30°C than at 10 or 20°C.	Nalewaja & Woznica, 1985
	Inverse relationship between sulfonylurea persistence and temperature.	Chlorsulfuron persistence decreased when soil temp increased from 20 – 40°C.	Anderson & Barrett, 1985
		Increasing temp. from 8-24°C reduced bioactivity of metsulfuron in a loam soil.	Anderson, 1985
Soil organic matter	Inverse relationship between sulfonylurea phytotoxicity and organic matter.	Greater injury to corn & sorghum from chlorsulfuron in soils with 2.5% organic matter than with 3.5%.	Peterson & Arnold, 1985
		Phytotoxic effects of primisulfuron methyl and metsulfuron methyl were not seen in mustard and sorghum 6 weeks after spraying in soils with 7.3% organic matter	James *et al.*, 1995

Table 2.4: Factors affecting the persistence of ALS-inhibiting herbicides in soils.

Environmental factors	Mechanism	Examples	References
		Chlorsulfuron persistence was longer in soils with 7.0% organic matter than in soils with 3.5% organic matter.	Junnila *et al.*, 1994
	Humic acid and iron oxides were important adsorbents of chlorsulfuron.	Chlorsulfuron adsorption on these adsorbents increased when pH increased from 4-8.	Shea, 1986; Mersie & Foy, 1985; Thirunarayanan *et al.*, 1985
	Soil adsorption of chlorsulfuron was directly related to soil organic matter.		Walker *et al.*, 1989
	Persistence of flumetsulam decreased with decreasing organic matter content.	When organic matter increased from1.2% to 3.5% persistence increased.	Shaw and Murphy, 1997
Soil Texture	Sulfonylureas persist longer in heavy textured soils	Sugarbeet, lentil and sunflower were less damaged in a sandy clay loam, than in a silty clay loam or sandy loam.	Beyer *et al.*, 1987 Kotoula-Syka *et al.*, 1993a and b
	Persistence may be directly affected by the influence of clay on soil water holding capacity and water movement.	Chlorsulfuron persisted longer in soils with clay content of 65.7% compared to those of 42.8% and 22.0%.	Vicari *et al.*, 1994

2.4 MICROBIAL TRANSFORMATION AND DEGRADATION

Warm, moist soil conditions promote microbial activity and these also promote sulfonylurea degradation (Shaw and Murphy, 1997; Lehmann *et al.*, 1993; Flint and Witt, 1991; Beyer *et al.*, 1985; Joshi *et al.*, 1985). Joshi *et al.* (1985) conducted experiments on the degradation of chlorsulfuron, and found that in acidic soils degradation proceeded by both hydrolysis and microbial breakdown, but in alkaline soils degradation is primarily by microbial breakdown. Degradation was 10-12 times faster in biologically active soils than soils sterilised by gamma irradiation or ethylene dioxide and the authors isolated a soil actinomycete (*Streptomyces griseolus)* and soil fungi (*Aspergillus niger* and *Penicillium sp)* capable of degrading ^{14}C – chlorsulfuron in pure culture (Joshi *et al.*, 1985). Ten weeks after application, microbial breakdown accounted for 79% of chlorsulfuron transformation in a biologically active silt loam and 91% in an alkaline silt loam (Beyer *et al.*, 1987). The number of days taken to break down 50% of thifensulfuron methyl increased from 0.75 days – 3.75 days in biologically active soils to 50 – 70 days in autoclaved soil (Brown *et al.*, 1997). The authors noted that the rapid de-esterification of thifensulfuron methyl to the herbicidally inactive thifensulfuron acid was at least partly due to microbial extracellular enzyme activity (Brown *et al.,* 1997).

Thus, microbial degradation is the main route of flumetsulam breakdown, but adsorption of the herbicide to the soil can reduce its availability to soil microbes (Lehmann *et al.*, 1993). Imazaquin and imazethapyr were almost completely degraded five months after application in a non sterile soil, while in the biologically inactive soil the herbicides had only been reduced by 14% (Flint and Witt, 1997).

2.5 EFFECTS OF ALS-INHIBITING HERBICIDES ON NON-LEGUME CROPS

As mentioned previously the persistence of ALS-inhibiting herbicides is influenced by soil pH, temperature, soil moisture, organic matter and soil type. Persistence is beneficial in terms of extended weed control but may cause damage to subsequent crops. Many studies have been conducted to investigate the effects of these herbicides on commercial crops and the weeds they are intended to control (Table 2.5).

Crop selective sulfonylureas have been commercialised for use in wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rice, corn (*Zea mays*), soybeans (*Glycine max*) and canola (*Brassica napus*) with additional crop selective herbicides for use in cotton, potatoes and sugarbeet (Brown, 1990). It is likely that any negative impact on these crops from the selective herbicides will be minimal (Brown, 1990).

The response of crops to ALS-inhibiting herbicides varies with the crop, soil type, pH, herbicide and time of application. Table 2.5 summarises the findings from various authors for the effects on non-leguminous crops. The effect on legumes will be discussed later in the review.

Table 2.5: Effects of ALS-inhibiting herbicides on non-leguminous crops (concurrent refers to effects observed in year of application. NA indicates information unavailable).

crop	herbicide	country	soil type	pH	time after application	injury (+/-)	author
Wheat various cultivars	chlorsulfuron (30g ha^{-1})	Australia (WA)	sandy loam	6.0	concurrent	Sensitive cultivars had inhibition of third leaf of seedlings	Bowran and Blacklow, 1987.
	chlorsulfuron (7.5, 15, 30 g ha^{-1})	Australia (WA)	sandy loam	5.8	concurrent	nutrient uptake affected	Osborne *et al.*, 1993
	chlorsulfuron (20g ha^{-1})	Australia (SA)	NA	NA	concurrent	yield decline with most varieties, although varied with season	Wheeler *et al.*, 1996
	chlorsulfuron (15 and 40 g ai ha^{-1})	Australia (NSW)	clay loam	4.1 - 4.8	concurrent	no yield loss	Lemerle *et al.*, 1985
	Chlorsulfuron (15 g ha^{-1})	New Zealand	NA	NA	concurrent	nitrate accumulation and growth inhibition	Andrews *et al.*, 1993; Dastgheib *et al.*, 1993
	chlorsulfuron (35,70,140,280, 560 g ha^{-1})	USA	silt loam	5.8	concurrent	visibly injured and reduction in height, grain yields not affected	Brewster & Appleby, 1983.
	chlorsulfuron + metsulfuron (17.5+3.5g ha^{-1}) triasulfuron + metribuzin (30 + 158g ha^{-1})	USA	fine loam - coarse loam	5.7 - 6.4	concurrent	no visible injury; fall in forage production	Koscelny *et al.*, 1996
	triasulfuron (35g ha^{-1})	Australia (SA)	NA	NA	concurrent	yield decline, season dependant	Wheeler *et al.*, 1996
	imazethapyr (70, 100, 200 g ha-1)	Canada	7.4-8.0		1 year	yield affected	Moyer & Esau, 1996

Table 2.5 cont..

crop	Herbicide	country	soil type	pH	time after application	injury (+/-)	author
Barley	metsulfuron methyl (4.2 & 8.4 g ha-1)	Australia (SA)		6.6 - 8.4	concurrent	temp. red^{n}. in uptake of P, Zn, Mn, Cu, S & K. Increase in grain protein at harvest	Pederson *et al.*, 1994
	chlorsulfuron (15g ai ha $^{-1}$)	Australia (SA)	NA	NA	concurrent	yield reduction	Wheeler *et al*, 1996
	chlorsulfuron (15 g ai ha $^{-1}$)	Australia (NSW)		4.3 - 6.9	concurrent	yield reduction	Lemerle *et al.*, 1990
	Chlorsulfuron	Canada	NA	8.0	2 years	yield affected	Moyer *et al.*, 1990
	triasulfuron (35g ha^{-1})	Australia (NSW)	NA	NA	concurrent	Yield reduction	Wheeler *et al*, 1996
	flumetsulam (25 & 50 g ha^{-1})	Australia (NSW)	NA	NA	concurrent	yield affected	Wheeler *et al*, 1996
Maize/ corn	chlorsulfuron (40g ai ha^{-1})	Greece	sandy loam, sandy clay loam, silty clay loam	4.7 - 7.9	8 months	no effect	Eleftherohorinos & Kotoula-Syka, 1989
	chlorsulfuron (40g ai ha^{-1})	Greece	sandy loam, sandy clay loam, silty clay loam	4.7 - 7.9	4 months	yield reduction	Eleftherohorinos & Kotoula-Syka, 1989
	chlorsulfuron (35,70,140,280560 g ha^{-1})	USA	silt loam	5.8	165 days	reduced growth	Brewster & Appleby 1983
	chlorsulfuron (17g ai ha^{-1})	USA	silty clay loam	6.5	24 months	significant injury	Petersen & Arnold 1985
	chlorsulfuron (22g ai ha^{-1})	Canada	fine sandy loam	7.4	1 year	visible injury	Friesen & Wall 1991

Table 2.5 cont..

crop	Herbicide	country	soil type	pH	time after application	injury (+/-)	author
Maize/corn	chlorsulfuron (0.00048 - 1.5 mg L^{-1}) metsulfuron methyl(0.00048 - 1.5 mg L^{-1})	Germany	growth media	NA	concurrent	72% & 55% respectively. Reduction in young primary roots	Flaburiari & Kristen 1996
	primisulfuron (13 g ai ha^{-1})	USA	NA	NA	concurrent	no injury	Frazier *et al.*, 1993
	primisulfuron + Terbufos (13 g ai ha^{-1} + 8 g/ha)	USA	NA	NA	concurrent	shoot dry wt & length reduced	Frazier *et al.*, 1993
	imazethapyr (70, 100 and 200 g/ha)	Canada		7.4 - 8.0	1 year	yield reduction	Moyer & Esau 1996
Sunflower	chlorsulfuron (10, 20, 40 g ai ha^{-1}), metsulfuron (10, 20, 40 g ai ha^{-1}), triasulfuron (10, 20, 40 g ai ha^{-1}),	Greece	sandy loam sandy clay loam silty clay loam	7.9 4.7 7.6	4 months	significant injury	Kotoula-Syka *et al.*, 1993b
	chlorsulfuron (10, 20, 40 g ai ha^{-1}), metsulfuron (10, 20, 40 g ai ha^{-1}), triasulfuron (10, 20, 40 g ai ha^{-1}),	Greece	sandy loam sandy clay loam silty clay loam	7.9 4.7 7.6	8 months	no injury	Kotoula-Syka *et al.*, 1993b
	chlorsulfuron (22g ai ha^{-1})	Canada	fine sandy loam	7.4	4 years	significant injury	Friesen & Wall 1991
	chlorsulfuron (22g ai ha^{-1})	Canada	clay loam	6.5	3 years	significant injury	Friesen & Wall 1991
	chlorsulfuron (17g ai ha^{-1})	USA	silty clay loam	6.5	24 months	significant injury	Petersen & Arnold 1985

Table 2.5 cont..

Crop	Herbicide	country	Soil type	pH	time after application	injury (+/-)	author
Sunflower	Imazethapyr	Canada	NA	7.4 - 8.0	1 year	yield reduced	Moyer & Esau, 1996
Canola	chlorsulfuron (22g ai ha^{-1})	Canada	fine sandy loam	7.4	3 years	significant injury	Friesen & Wall, 1991
	chlorsulfuron (22g ai ha^{-1})	Canada	clay loam	6.5	3 years		Friesen & Wall, 1991
	chlorsulfuron (17g ai ha^{-1})	USA	silty clay loam	6.5	24 months		Petersen & Arnold, 1985
	chlorsulfuron (10, 20, 40 g ha^{-1})	Canada	NA	8.0	3 years	significant injury	Moyer *et al.*, 1990
	thifensulfuron & thifensulfuron: tribenuron (0.1 g ai ha^{-1})	Canada	clay loam Heavy clay	7.4 8.0	concurrent	delayed flowering; reduced yield & seed germination	Wall *et al.*, 1995
	chlorsulfuron (17g ai ha^{-1})	USA	silty clay loam	6.5	24 months	significant injury	Petersen & Arnold, 1985
Mustard	imazethapyr (70, 100, 200 g ha^{-1})	Canada	NA	7.4 - 8.0	1 year	yield reduced	Moyer & Esau, 1996
Sugarbeet	chlorsulfuron (10, 20, 40 g ha^{-1})	Canada	NA	8.0	6 years	significant injury	Moyer *et al.*, 1990
Sugarbeet	chlorsulfuron, metsulfuron, triasulfuron (10, 20, 40 g ha^{-1})	Greece	sandy loam sandy clay loam silty clay loam	7.9 4.7 7.6	4 months	significant injury	Kotoula-Syka *et al.*, 1993b
Sugarbeet	chlorsulfuron, metsulfuron, triasulfuron (10, 20, 40 g ha^{-1})	Greece	sandy loam sandy clay loam silty clay loam	7.9 4.7 7.6	8 months	no injury	Kotoula-Syka *et al.* 1993b

2.6 EFFECTS OF ALS-INHIBITING HERBICIDE RESIDUES ON LEGUMES AND SYMBIOTIC NITROGEN FIXATION

2.6.1 Introduction

Biological nitrogen fixation (BNF) is a property unique in nature possessed by only a few genera of prokaryotic organisms (Havelka *et al.*, 1982; Brady, 1990). Organisms capable of fixing nitrogen contain the genetic information required to synthesise the enzyme nitrogenase which catalyses the process of nitrogen fixation (Havelka *et al.*, 1982; Brady, 1990). Nitrogen fixation is defined as the process whereby atmospheric nitrogen is reduced to ammonia and therefore becomes available as a nutrient for other organisms (Brady, 1990; Halveka *et al.*, 1982). The symbiosis that occurs between legume plants and bacteria of the genera *Rhizobium, Bradyrhizobium and Sinorhizobium* provides the major source of biologically fixed nitrogen in agricultural soils (Vance, 1997; Brady, 1990). Although legumes are heavy users of nitrogen (with a foliage content of 2-4% N and seed protein contents of 17-40%), they can potentially obtain their full nitrogen requirements via their symbiosis with rhizobia (Peoples *et al.,* 1992). This symbiosis is used in certain agricultural systems to replenish soil N and augment the supply of nitrogen to subsequent non-legume crops (Coventry and Evans, 1989). A review by Peoples *et al.* (1992) stated that 40 – 80 kg N ha^{-1} accrues annually under subterranean clover in rain-fed systems of southern Australia. Soil mineral nitrogen generated under grain legume crops range from 20 – 80 kg N ha^{-1} (Evans *et al.*, 1991; Unkovich *et al.*, 1997).

2.6.2 Nodule formation

In order to achieve successful nodulation, *Rhizobium* must be present in the free living form in the root rhizosphere (Richardson *et al.*, 1989). The process of nodule formation is presented in Figure 2.1 (Richardson *et al.*, 1989). Legume plants release root exudates in the zone of pre-emergent root hair cells (Schmidt *et al.*, 1994; Bhuvaneswari *et al.*, 1980; Bhuvaneswari *et al.*, 1981) that stimulate the growth of rhizobia near the root surface (Bowen and Rovira, 1976). Root exudates contain specific flavonoid compounds that induce the expression of nodulation genes in *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* spp. (Stacey *et al.*, 1995; Brewin *et al.*, 1993; Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986). Flavonoid accumulation in the rhizosphere is induced by Nod factors (rhizobial lipooligosaccharide signals) from the bacteria (Schmidt *et al.*, 1994). The rhizobia cells then attach or clump to the developing root hair tips (Dazzo, 1984). This attachment can happen seconds or minutes after the introduction of rhizobia to the rhizosphere (Bauer, 1981) and induces root hair curling and branching (Bauer 1981; Richardson *et al.*, 1989). The process of root hair curling, however, is not fully understood. Yao and Vincent (1976) found that root hair curling only occurred when specific *Rhizobium* were present. The substances present in rhizobia that induce these root hair deformations are controlled by bacterial *nod* genes and are known as Nod promoters (Suominen *et al.*, 1999; Felle *et al.*, 1995; Roche *et al.*, 1991). Once the root hair begins to curl, signals are 'sent' from the bacteria to the root cortex and cortex cells begin to differentiate, eventually forming the nodule meristem (Richardson *et al.,* 1989). Rhizobia bacteria then invade the curling root hair cells probably by enzymatic degradation of the cell wall (Callaham and Torrey, 1981). Cárdenas *et al.* (1998) found that Nod factors alter the organisation of actin microfilaments in root hair cells and hypothesise that this is a

prelude to the formation of infection threads. Infection threads carry the bacteria to the developing nodule meristem (Downie and Johnston, 1986; Bauer, 1981; Callaham and Torrey, 1981). The rhizobia are released from the thread upon arrival at the meristem and differentiate into forms that are capable of fixing nitrogen (Richardson *et al.*, 1989; Downie and Johnston, 1986; Bauer, 1981). Both the bacteria and the plant supply gene products that are necessary for nitrogen fixation. For example, the bacteria will provide nitrogenase and the plant supplies leghemoglogin and glutamine synthetase (Downie and Johnston, 1986).

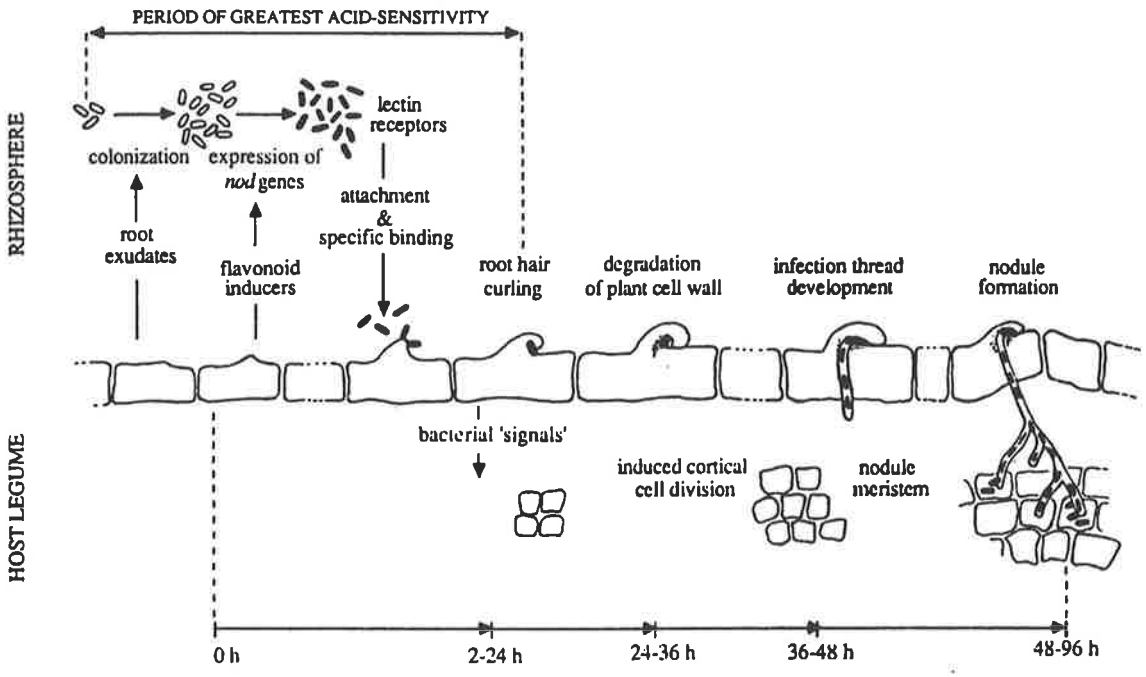


Figure 2.1: Schematic diagram representing infection of root cells by rhizobia and subsequent nodule formation (taken from Richardson *et al.*, 1989).

2.6.3 Benefits of legumes and nitrogen fixation to the farming system

The balance between the input of nitrogen (generally through BNF) and the loss through removal, leaching or gaseous emissions is a critical component in the ability of Australian soils to sustain pasture and crop production (Fillery, 1992). Sustainability in agriculture is becoming as desirable as maintaining productivity (Giller and Cadisch

1995; Peoples *et al.*, 1995 a, b and c) and in order for a farming system to remain sustainable it is necessary to replenish nutrients lost from the soil (Peoples *et al.*, 1995a, b and c). One of the driving forces behind agricultural sustainability is the management of nitrogen in the environment (Vance, 1997). The use of nitrogen fixing species in cropping systems may reduce the need for nitrogen fertilisers and increase soil health (Vance, 1997; Peoples *et al.*, 1995b). Many factors including physical, environmental and biological factors and nutrient availability may influence nitrogen fixation (Peoples *et al.*, 1992; Bergersen *et al.*, 1989; Coventry and Evans, 1989). Therefore nitrogen fixation may be open to manipulation and improvement (Vance, 1997; Peoples *et al.*, 1995c; Peoples *et al.*, 1992; Bergersen *et al.*, 1989). Consequently, it should be possible to manage nitrogen fixation better to aid in sustainability and reduce fertiliser inputs (Peoples *et al.*, 1995a, b and c).

If an effective symbiosis is not achieved in soils low in mineral nitrogen a loss of legume production will be observed (Peoples et al., 1989). Nitrogen fertiliser applications of up to 160 kg N ha^{-1} may be required to achieve seed yields similar to those of a well-nodulated soybean crop (Gault *et al.*, 1984). Legumes can compensate for poor nitrogen fixation by using nitrogen from the soil when soil mineral nitrogen levels are sufficient, resulting in an exploitation of nitrogen reserves. In such situations soil nitrogen fertility is lost, which represents an inefficient use of a legume in a cropping sequence (Peoples *et al.*, 1989).

The benefits of including legumes in a crop rotation include:

1. Improved soil structure and increased nutrient availability from legume residues;
2. Breaking disease and cereal pest cycles;
3. Increased soil microbial activity following addition of legume residues;
4. Increased levels of nitrate remaining in the soil when compared to cereal crops (Peoples *et al.*, 1992).

The importance of biological nitrogen fixation in crop rotation systems has increased in response to increased costs of nitrogenous fertilisers (Eberbach, 1993). Annual accretions of 40-80 kg N ha^{-1} have been reported under subterranean clover in rain fed systems of South Australia, although even higher values (>100kg N ha^{-1} yr^{-1}) may occur with irrigation or under lucerne (Peoples *et al.*, 1992). Estimates of the proportion of fixed nitrogen vary with legume species, soil nitrogen status, water supply, grazing, or with season, pasture age and composition (Peoples *et al.*, 1992). The proportion of nitrogen fixed for legume crops ranges from 6 to 97% equating to 29 to 348 kg N ha^{-1} $crop^{-1}$ (Peoples *et al.*, 1995a, b and c). The proportion of nitrogen fixed by narrow leaf lupin, chickpea and field pea grown in NSW was 83%, 76% and 60 % respectively (Armstrong *et al.*, 1997). In NSW lupins had the potential to contribute an average of 40.3 kg ha^{-1} (range – 41 to 135 kg ha^{-1}) to soil N (Evans *et al.*, 1989). In Western Australia, predicted nitrogen returns to the soil from fixed nitrogen from lupins averaged 65 kg ha^{-1} (range 32-96 kg ha^{-1}) (Unkovich *et al.*, 1994).

In the USA, new and novel uses for legumes are being developed and include: (i) development of nitrogen fixing plants to aid in phytoremediation of contaminated

sites (e.g. lucerne to take up excess nitrate and atrazine from the soil); (ii) growth of legumes for generation of electrical energy; and (iii) production of industrial and pharmacological products (Vance, 1997). These new and broader roles may encourage greater usage of legumes in the farming system with a range of options available for the farmer.

2.6.4 Herbicides and nitrogen fixation

Herbicide usage in grain and pasture legumes reduces competition from weeds resulting in greater biomass production and yield, increased returns of biologically fixed nitrogen and thereby improved benefits to the nitrogen budget of the soil. Many herbicides are registered for use in grain and pasture legumes including the ALS-inhibiting herbicides Broadstrike (flumetsulam) and Spinnaker (imazethapyr). If a herbicide is toxic to the legume plant or the legume-rhizobia symbiosis (either from direct application or residual), reduced plant yield or a reduction in the input of nitrogen to the soil could result (Eberbach, 1993). However, losses in nitrogen fixation can be prevented by altering management practices, such as the use of tolerant legumes and changing herbicide usage (Eberbach, 1993).

Herbicides may affect the legume-*Rhizobium* symbiosis in a number of ways including: (i) direct effects on the host plant; (ii) by reducing survival or growth of rhizobia; (iii) through inhibition of the nodulation process; or (iv) by influencing nitrogen fixation (Eberbach, 1993). The effects of ALS-inhibiting herbicides on these four processes will be discussed in the following pages.

2.6.5 Effects of ALS-inhibiting herbicides on the host plant

Many legumes appear to be sensitive to the ALS-inhibiting herbicides, particularly sulfonylureas, and show injuries that are more pronounced and persist longer than for non-leguminous crops. Evidence that ALS-inhibiting herbicides inhibit the growth of legumes has been reported by a number of authors (Table 2.6).

Lentils (*Lens culinaris*) appear to be particularly sensitive and have shown injury symptoms from applications of chlorsulfuron, metsulfuron methyl, triasulfuron (Friesen and Wall, 1991; Moyer *et al.*, 1990) and a mixture of thifensulfuron and tribenuron (Wall, 1994). Seven years were required before no yield loss was observed in lentils following a chlorsulfuron application on a soil of pH 8.0 (Table 2.6) (Moyer *et al.*, 1990). Other crops such as field pea *(Pisum sativum*), soybean (*Glycine max*), lucerne (*Medicago sativa*) and chickpea (*Cicer arietinum*) also exhibit symptoms of phytotoxicity from chlorsulfuron residues and details are given in Table 2.6. Phytotoxicity from ALS-inhibiting herbicides can also be found in pasture species such as medic and subterranean clover (Table 2.6) (Fajri *et al.,* 1996; Gillett and Holloway, 1996; Evans *et al.,* 1993; Rovira *et al.* 1993).

It appears that crop and pasture cultivars differ in their responses to these herbicides (Fajri *et al.*, 1996). The subterranean clover cultivar Junee was sensitive to flumetsulam, imazethapyr and mixtures of imazethapyr with diuron and metribuzin, but not to a mixture of imazethapyr and simazine (Table 2.6) (Fajri *et al.,* 1996).

It appears that the imidazolinone and sulfonamide herbicides are less damaging to legumes than the sulfonylureas (Table 2.6). Chickpea yields were unaffected by imazethapyr or flumetsulam residues in soils of the Darling Downs regions of Queensland with pH ranging from 5.5 - 7.8 (Table 2.6) (Barnes *et al.,* 1996).

Flumetsulam successfully removed weeds from medic and clover pastures, resulting in greater seed set and significant increases in wheat yield and protein one year after legume pasture (Gilmour, 1996). The increase in pasture seed set was likely due to reduced weed competition. Lentils and chickpea in South Australia were tolerant to flumetsulam, although dry conditions gave poor chickpea yields and further research is required (Table 2.6) (Wheeler *et al.*, 1996). Peas were generally tolerant to both imazethapyr and flumetsulam in the same South Australian trial, although imazethapyr applied post-sowing/pre-emergence appeared safe in one year, but caused a yield loss in another (Table 2.6) (Wheeler *et al.*, 1996). It is possible that climatic factors in different years cause a variation in crop responses to this herbicide. Observations of initial leaf discolouration and stunting of growth have been observed on chickpeas and medic following the application of flumetsulam and imazethapyr (Cyanamid herbicide label; DowElanco herbicide label). Whether such a phytotoxic effect has an influence on nodulation and nitrogen fixation by legumes has not been investigated.

Table 2.6: Effects of ALS-inhibiting herbicides on legume crops and pastures (NA = not available; ID50 = 50% inhibition of growth).

crop/pasture	herbicide	country	soil type	pH	time after application	injury	author
Soybeans	chlorsulfuron (17, 34, 68 g ha^{-1})	USA	silty clay loam	5.3 - 6.5	12 months 24 months	significant injury no injury	Petersen & Arnold, 1985
	chlorsulfuron (9, 18 and 36 g ha^{-1})	USA	silt loam	6.3	12, 24, 36 months	36 g ha^{-1} reduced yield when applied pre-emergence to wheat	Ritter *et al.*, 1988
	primisulfuron	USA	silt loam	6.4 - 6.6	concurrent (drift)	chlorosis, necrosis, yield loss	Bailey & Kaputsa, 1993
Field pea	chlorsulfuron (22 g ai ha^{-1})	Canada	fine sandy loam	7.4 and 6.5	12 months	visible injury	Friesen & Wall, 1991; Moyer *et al.*, 1990
	chlorsulfuron (10, 20,40 g ha^{-1}))	Canada	NA	8.0	3 years	injury visible until this time	Moyer *et al.*, 1990
	Chlorsulfuron (10, 20,40 g ha^{-1})	Greece	Clay; silty clay	6.6 - 7.4	8 - 14 months	decrease in no. of plants/m^2; dry matter weights; grain yield	Efthimiadis *et al.*, 1989)
	triasulfuron	Greece	clay; silty clay	6.6 - 7.4	8 - 14 months	tolerant	
	thifensulfuron : tribenuron)	Canada	clay loam	6.5	Concurrent (drift)	37% yield loss	Wall, 1994
	Flumetsulam (25 & 50 g ha^{-1}), imazethapyr	Australia (SA)	NA	NA	concurrent	safe	Wheeler *et al.*, 1996

Table 2.6 cont..

crop/pasture	herbicide	country	soil type	pH	time after application	injury	author
Lentil	chlorsulfuron (22 g ai ha $^{-1}$)	Canada	clay loam	6.5	3 years	injury up to 3 years	Friesen & Wall, 1991
	chlorsulfuron (10, 20, 40 g ha^{-1})	Canada	NA	8.0	7 years	injury observed up to 7 years	Moyer *et al.*, 1990
	chlorsulfuron	Greece	clay; silty clay	6.6 - 7.4	8 - 14 months	decrease in no. of plants/m^2; shoot dry matter; grain yield	Efthimiadis *et al.*, 1989
	triasulfuron	Greece	clay; silty clay	6.6 - 7.4	8 - 14 months	tolerant	
	chlorsulfuron, metsulfuron, triasulfuron (10, 20, 40 g ha^{-1})	Greece	sandy loam	7.9	4 months	significant injury	Kotoula-Syka *et al.*, 1993
			sandy clay loam	4.7		" "	
			silty clay loam	7.6		" "	
	chlorsulfuron, metsulfuron, triasulfuron (10, 20, 40 g ha^{-1})	Greece	sandy loam	7.9	8 months	significant injury	Kotoula-Syka *et al.*, 1993b
			sandy clay loam	4.7		no injury	
			silty clay loam	7.6		significant injury	
	Thifensulfuron	Canada	clay loam	6.5	Concurrent (drift)	22% yield reduction	Wall, 1994
	Flumetsulam (25 & 50 g ha^{-1})	Australia (SA)	NA	NA	concurrent	safe	Wheeler *et al.*, 1996

Table 2.6 cont..

crop/pasture	herbicide	country	soil type	pH	time after application	injury	author
Chickpea (Barwon) (Amethyst)	chlorsulfuron (0.05 - 1000 ug ai L^{-1})	Australia (QLD)	soil free system	NA	concurrent (residual levels)	ID50 = 1.01 ID50 = 2.54	Churchett *et al.*, 1996
Chickpea	chlorsulfuron	Australia (NSW)	NA	NA	12 months	yellowing of shoot tips; inhibition of root growth; yield reduction; premature death	Ferris *et al.*, 1992
Chickpea	chlorsulfuron	Greece	clay; silty clay	6.6 - 7.4	8 - 14 months	decrease in no. of plants/m^2; shoot dry matter; grain yield	Efthimiadis *et al.*, 1989)
Chickpea (Amethyst)	metsulfuron (0.05 - 1000 ug ai L^{-1})	Australia	soil free system	NA	concurrent (residual levels)	ID50 = 3.73	Churchett *et al.*, 1996
Chickpea (Amethyst)	triasulfuron (0.05 - 1000 ug ai L^{-1})	Australia	soil free system	NA	concurrent (residual levels)	ID50 = 1.27	Churchett *et al.*, 1996
	triasulfuron	Greece	clay; silty clay	6.6 - 7.4	8 - 14 months	tolerant	Efthimiadis *et al.*,1989
Chickpea	flumetsulam (25 & 50 g ha^{-1})	Australia (SA)	NA	NA	concurrent	tolerant	Wheeler *et al.*, 1996
Chickpea	imazethapyr (72 g ai ha^{-1}), flumetsulam (20 g ai ha^{-1})	Australia	various	5.5 - 7.8	time of application	unaffected	Barnes *et al.*, 1996

Table 2.6 cont..

crop/pasture	herbicide	country	soil type	pH	time after application	injury	author
Alfalfa	chlorsulfuron (35, 70, 140, 280, 560g ha^{-1})	USA	silt loam	5.8	275 days	growth inhibition	Brewster & Appleby, 1983
	chlorsulfuron (10, 20, 40 g ha^{-1})	Canada	NA	8.0	6 years	recovered after this time	Moyer *et al.*, 1990
Bean	chlorsulfuron (10, 20, 40 g ha^{-1})	Canada	NA	8.0	4 years	recovered after this time	Moyer *et al.*, 1990
Cowpea	Imazethapyr	USA	silt loam	6.5	concurrent	tolerant	Baerg & Barrett, 1996
Medic	chlorsulfuron (11 g ai ha^{-1})	Australia (SA)	light sandy clay loam	8.5 - 9.5	12 months	reduction in number, dry matter and seed yield	Evans *et al.*, 1993; Rovira *et al.*, 1993
Medic	triasulfuron (21 g ai ha^{-1}); (0.5, 2 ug kg^{-1})	Australia (SA)	light sandy clay loam	8.5 - 9.5	12 months	decrease herbage; decrease seed production	Evans *et al.*, 1993; Gillett & Holloway, 1996
	metsulfuron (4 g ai ha^{-1})	Australia	light sandy clay loam	8.5 - 9.5	12 months	reduction in seed yield	Evans *et al.*, 1993
Clover	chlorsulfuron (0.2 mg ai L^{-1})		sandy loam		4 months	seedlings affected	Eberbach & Douglas, 1991
	flumetsulam (25g ha^{-1})	Australia (WA)	NA	NA	Concurrent	tolerant	Gilmour, 1996
	flumetsulam+ diuron (25g ha^{-1} + 100ml)	Australia (WA)	NA	NA	concurrent	tolerant	Gilmour, 1996
Subterranean clover (Junee)	flumetsulam (25g) imazethapyr (200ml) imazethapyr (150ml)				time of application	Sensitive Sensitive Sensitive	Fajri *et al.*, 1996

2.6.6 Effects of ALS-inhibiting herbicides on survival and growth of rhizobia

Generally, herbicides (when applied at recommended rates) show no effect on total numbers of bacteria in the soil (Eberbach, 1993; Moorman, 1989; Anderson, 1978). Effects of herbicides on bacteria are often short-lived with subsequent recovery or slightly greater than normal populations within a short period (Anderson, 1978). Many studies investigating herbicidal effects on rhizobia use unrealistically high levels of the herbicide. Relatively little work has investigated the effects ALS-inhibiting herbicides on the survival and growth of rhizobia. As bacteria possess the ALS enzyme (Hershey *et al.*, 1999; Duggleby, 1997; Xing and Whitman, 1994; LaRossa *et al.*, 1987), it is possible that these herbicides may have an effect on rhizobial growth or cell biochemistry. A summary of research is presented in Table 2.7.

Growth of *Rhizobium meliloti* strain 14 and *Rhizobium leguminosarum* bv *trifolii* in pure culture were unaffected by chlorsulfuron at rates corresponding to 50 and 500 times the recommended field application rate for Sweden (Martensson and Nilsson, 1989). *Rhizobium trifolii* sustained relatively normal growth in the presence of low levels of chlorsulfuron (0.2, 0.5 and 1.0 mg ai L^{-1} in pure culture), but when the concentration was increased to 2 mg ai L^{-1}, growth was inhibited by approximately 40% (Eberbach and Douglas, 1989). Different species of *Rhizobium leguminosarum* bv *trifolii*, *R. Meliloti* and *R. loti* varied in sensitivity to chlorsulfuron with some showing tolerance and some sensitivity (Martensson, 1992).

Gonzalez *et al.* (1996) grew *Rhizobium leguminosarum* bv *viciae* strain NLV8 in pure culture on a complex growth medium containing imazethapyr at levels between 14 and 7000 times the recommended field application rate in Spain. Bacterial growth was only affected at the very high doses.

It should be pointed out that all of these studies were carried out in the laboratory and soil conditions were not taken into account. However, from these results it is likely that under field conditions and at recommended application rates, these herbicides will have little effect on *Rhizobium* growth or population size.

Table 2.7: The effects of ALS-inhibiting herbicides on *Rhizobium*.

plant species	*Rhizobium* sp	herbicide	application rate	effect	country	author
Alfalfa (*Medicago sativa)*	*R. meliloti* strain 14	chlorsulfuron	0.55 and 5.5 uM (50 & 500 times recommended. rate)	unaffected	Sweden	Martensson & Nilsson, 1989
Red Clover *(Trifolium pratense)*	*R. leguminosarum* bv *trifolii*	chlorsulfuron	0.55 and 5.5 uM (50 & 500 times recommended. rate)	unaffected	Sweden	Martensson & Nilsson, 1989
Pea (*Pisum sativum*)	*R. leguminosarum* bv *viciae*	imazethapyr	0.34mM - 3.4mM	unaffected except at high doses	Spain	Gonzalez *et al.*, 1996
Sub clover (*Trifolium subterraneum)*	*R. trifolii*	chlorsulfuron	0.2, 0.5, 1.0 mg L -1 nutrient solution	unaffected i.e. normal growth	Australia (NSW)	Eberbach & Douglas, 1989
Sub clover (*Trifolium subterraneum)*	*R. trifolii*	chlorsulfuron	2.0 mg L-1	growth inhibited	Australia (NSW)	Eberbach & Douglas, 1989
Red Clover*(Trifolium pratense)*	*R. leguminosarum* bv *trifolii*	chlorsulfuron	1.0 to 10 mg L^{-1}	sensitive (depending on strain)	Sweden	Martensson, 1992
Lucerne (*Medicago sativa)*	*R. meliloti*	chlorsulfuron	1.0 to 10 mg L^{-1}	sensitive (depending on strain)	Sweden	Martensson, 1992

2.6.7 Effects of ALS-inhibiting herbicides on the nodulation process

Although herbicides may not affect the growth and survival of rhizobia, it is possible that some stage of the nodule formation process may be affected. Again, there is limited evidence of the effects of ALS-inhibiting herbicides on nodulation. This research has been summarised in Table 2.8.

Chlorsulfuron at concentrations indicative of that remaining in soil twelve months after application (0.28 pM) inhibited early root hair infections of alfalfa in growth media (Table 2.8) (Martensson and Nilsson, 1989). Nodules did not develop on lucerne plants grown in soil with additions of various levels of chlorsulfuron (Martensson and Nilsson, 1989). Chlorsulfuron at concentrations ranging from 2 - 20 mg L^{-1} caused severe reductions in nodule numbers in *Trifolium subterraneum* grown on agar slopes and inoculated with *Rhizobium trifolii* (Table 2.8) (Eberbach and Douglas, 1989). Chlorsulfuron reduced bacterial-induced root hair formations necessary for the formation of nodules in a number of legumes (Table 2.8) (Martensson, 1992). The number of nodules forming on a variety of legumes was inhibited by chlorsulfuron in a Swedish study (Table 2.8) (Martensson, 1992). Additionally, chlorsulfuron (5 g ha^{-1}) reduced lucerne growth and nodulation (Table 2.8) (Koopman *et al.*, 1995).

Flumetsulam, imazethapyr and imazethapyr in mixtures with diuron and metribuzin (Fajri *et al.*, 1996) significantly reduced numbers of nodules on several medic cultivars. In the same study, herbicide impact on nodule numbers in clover varied with the cultivar. Two cultivars (Clare and Rosedale) showed no negative effects from the above herbicides, while Dalkeith experienced a reduction in nodule numbers with flumetsulam or imazethapyr, but not with any of the imazethapyr mixtures (Table

2.8) (Fajri *et al.*, 1996). Gonzalez *et al.* (1996) found that imazethapyr reduced the number of nodules per plant rather than the size of nodules on peas in Spain, suggesting an effect on nodule initiation rather than on nodule development (Table 2.8).

These results suggest that ALS-inhibiting herbicides can inhibit the formation of nodules on legumes, even though rhizobial populations were not necessarily affected. Reduced nodulation of legumes would have a deleterious effect on nitrogen fixation and hence affect nitrogen fertility status of the soil.

Table 2.8: Effects of ALS-inhibiting herbicides on nodulation.

plant species	*Rhizobium* sp	herbicide	Application rate	effect	country	author
Alfalfa (*Medicago sativa)*	*R. meliloti* strain 14	chlorsulfuron	0.28pM 4 and 8 g ha^{-1}	Early root hair infections inhibited No nodule formation	Sweden	Martensson & Nilsson, 1989
Pea (*Pisum sativum*)	*R. leguminosarum* bv *viciae*	imazethapyr	> 1.73 uM	Number of nodules declined; size of nodules not affected	Spain	Gonzalez *et al.*, 1996
Sub clover (*Trifolium subterraneum)*	*R. trifolii*	chlorsulfuron	2 - 20 mg L^{-1}	Reduction in nodule number	Australia (NSW)	Eberbach & Douglas, 1989
Medic (various cultivars)		flumetsulam imazethapyr	25 g ha^{-1} 200 ml ha^{-1}	Nodule number. Significantly reduced by all herbicides	Australia (SA)	Fajri *et al.*, 1996
Clover (various cultivars)		flumetsulam imazethapyr	25 g ha^{-1} 200 ml ha^{-1}	Varied with cultivar	Australia (SA)	Fajri *et al.*, 1996
Red Clover*(Trifolium pratense)*	*R. leguminosarum* bv *trifolii*	chlorsulfuron	1.0 to 10 mg L^{-1}	Nodule formation did not occur at higher concentrations	Sweden	Martensson, 1992
Lucerne (*Medicago sativa)*	*R. meliloti*	chlorsulfuron	1.0 to 10 mg L^{-1}	Nodules did not occur at increasing concentrations	Sweden	Martensson, 1992
Lucerne (*Medicago sativa)*	*R. meliloti*	chlorsulfuron	5 and 10 g ha^{-1}	Reduced nodulation	Australia (SA)	Koopman *et al.*, 1995

2.6.8 The effect of ALS-inhibiting herbicides on nitrogen fixation

Relatively little work has investigated the influence of ALS-inhibiting herbicides on the process of nitrogen fixation within nodules. Chlorsulfuron inhibited the nitrogenase activity (measured by acetylene reduction) of lucerne nodules and this was believed to be a result of adverse effects on plant growth and development rather than on the rhizobia (Martensson and Nilsson, 1989). Imazethapyr also caused a decline in nitrogenase activity in peas, lupins and soybeans (Sawicka *et al.*, 1996).

These studies suggest that the process of nitrogen fixation may be affected by ALS-inhibiting herbicides. However, further research is required to find the extent of any inhibition of nitrogen fixation, particularly as the method of acetylene reduction used in both previous studies is now considered unreliable. The natural abundance N^{15} method is preferable to this and involves the use of natural isotopes of nitrogen (Peoples *et al.* ,1989).

2.7 SUMMARY AND CONCLUSIONS

Sulfonylurea herbicides, recommended for use in cereals, have been found to persist in alkaline soils and inhibit growth and yield of subsequent rotational crops and pastures (particularly legumes). Other ALS-inhibiting herbicides including imidazolinones and sulfonamides, although generally safer for legumes, may affect crops and pastures in the year of application. Injury levels vary with the crop, crop variety, pH, soil type and herbicide used. Tolerant crops appear to rapidly metabolise the herbicide to non-toxic by products.

When considering the important contribution made to the nitrogen balance of Australian farming systems by legumes, an inhibition of legume productivity by herbicides could lead to possible serious consequences. It is important to know the influence these herbicides are having on the legume-rhizobia symbiosis. Even though rhizobial populations appear to be unaffected by these herbicides, it appears that the host plant, nodulation and nitrogen fixation are affected in a number of legumes. At what stage is the problem occurring? Is the damage noticed in the host plant a result of insufficient nodulation or nitrogen fixation, or is it a result of direct inhibition on the growth of the plant? These questions, as yet, have not been adequately answered. Inhibition at any of the above stages, may impact on the level of nitrogen fixed by the plant. This will result in a reduction in legume biomass, a loss in soil nitrogen fertility for the following cereal crop and generally represents a wasteful use of a legume in a cropping sequence. Increased fertiliser nitrogen inputs would be required in the year subsequent to legume production, thereby increasing costs to farmers.

2.8 THESIS OBJECTIVES

The main objectives of this thesis were to:

1. Investigate the effects of selected ALS-inhibiting herbicides on the growth and productivity of a pasture and a grain legume;

2. Determine the effects of the ALS-inhibiting herbicides on nitrogen fixation;

3. Investigate the possible mechanisms responsible for any reduction in nitrogen fixation.

These possible mechanisms include:

(i) a direct effect on the host plant;

(ii) an effect on the growth of *Rhizobium*;

(iii) an impact on the numbers of nodules formed; and

(iv) a reduction in the rate of nitrogen fixed per unit of nodule mass.

Figure 1.1 (Chapter 1) outlined the structure this thesis will follow.

CHAPTER 3

THE EFFECTS OF FLUMETSULAM ON THE GROWTH OF CHICKPEAS – A GLASSHOUSE EXPERIMENT

3.1 INTRODUCTION

In recent times, the production of chickpeas has increased rapidly, particularly in drought restricted areas in Queensland and New South Wales (Siddique and Sykes, 1997). In 1996/97 12,000 hectares were sown and 17,000 tonnes of chickpeas were produced in South Australia (PIRSA, 1998). In Australia, the amount of nitrogen fixed by chickpeas has been shown to vary between 15 and 124 kg N ha^{-1} (Armstrong *et al.*, 1997; Horn *et al.*, 1996b; Evans *et al.*, 1989). Future expansion in Australian grain legume production is likely to be dominated by chickpea and faba bean (Siddique and Sykes, 1997).

Flumetsulam (an ALS-inhibiting herbicide) has been recommended for controlling weeds such as Indian hedge mustard (*Sisymbrium orientale*) and ball mustard (*Neslia paniculata*) in legume pastures such as medic, and also in grain legume crops such as field pea and chickpea (DowElanco, 1996; Chambers, 1995). There is, however, anecdotal evidence to suggest that applications of flumetsulam may lead to some yellowing and stunting of growth in medic pastures and field pea crops (DowElanco, 1996), although no previous studies have investigated the effects of this herbicide on chickpeas.

Selective herbicides can be used in grain legume crops to allow the control of grass weeds with herbicides, that aids in removal of cereal root pathogens and also reduces the numbers of weeds present in the subsequent cereal crop (Tow and Schultz, 1991). The glasshouse experiment described in this chapter was designed to determine the effects of flumetsulam on the growth and nodulation of chickpea. The aims of the experiment were:

1. To quantify the effect of flumetsulam on shoot and root biomass, nitrogen content and number of root nodules of chickpea plants;

2. To determine if the addition of nitrogen fertiliser could overcome any changes in measured parameters following flumetsulam application.

3.2 MATERIALS AND METHODS

3.2.1 Experimental set up

The experiment was undertaken in 4.5 L pots. The pots were lined with plastic bags to avoid any loss of the herbicide and filled with a soil mixture recommended for the growth of lucerne and medic. The soil consisted of equal parts of coarse white sand and grey sand. The sand was mixed with 0.8 g potassium sulphate, 0.8 g micromax trace elements, 0.25 g superphosphate and 0.5 g calcite fertilisers per litre. The mix was then pasteurised at 100°C for 30 minutes. Each pot contained 4.5 kg of soil.

Prior to planting, Desavic chickpea seeds were inoculated with Nitrogerm 100 Group N chickpea inoculant manufactured by Biocare Technology Pty. Ltd (Somersby, NSW). Ten seeds were sown into each pot and these were thinned to three seedlings

per pot after germination. A layer of white plastic beads was placed on top of the soil in each pot to prevent excessive surface drying and to minimise algal growth. The pots were arranged in a shaded and cooled glasshouse. A spray bottle was used to keep the soil moist until seedling emergence, after which all pots were watered weekly to 10% gravimetric water holding capacity.

3.2.2 Treatments

Six nitrogen treatments were established to investigate whether the addition of nitrogen fertiliser would help the plant overcome any herbicide effects observed. The rates of nitrogen application (applied as KNO_3) are summarised in Table 3.1.

The two herbicide treatments were with and without flumetsulam. The commercial formulation of flumetsulam, Broadstrike, was applied at a rate equivalent to the recommended application rate of 25 g ha^{-1}. It was applied to the chickpea seedlings at the 4 – 6 branch stage as per label recommendations using a gas powered 2m long hand held boom, calibrated at walking speed.

Table 3.1: Nitrogen treatments used in the experiment, showing equivalent field application rates in kg N ha^{-1}.

Nitrogen treatment	
Kg N ha^{-1}	**N g pot^{-1}**
0	0
10	0.045
20	0.090
30	0.135
50	0.225
100	0.450

3.2.3 Experimental design

The two herbicide treatments (sprayed (+), unsprayed (-)) were crossed with the six nitrogen application rates (0, 10, 20, 30, 50 and 100 kg ha^{-1}) and five replicates of

each treatment combination were organised into blocks. The experiment was set up in the glasshouse in a randomised block design, blocked by replicate. There were 4 harvest times: at the time of spraying, and at one, three and six weeks after spraying. The total of 240 pots was rotated around the glasshouse every two weeks to minimise site effects within the glasshouse. The results were analysed by an analysis of variance that included the main effects of herbicide, nitrogen and harvest time and their interactions, using the statistical program Genstat 5 Release 4.1 (Payne, 1993).

3.2.4 Plant dry matter and nodulation measurements

Chickpea shoot and root biomass were determined at each harvest time. Plant material was dried at 60°C for 48 hours to determine dry weight. The dry matter data was later used in calculating relative growth rates (RGR) (equation 3.1):

$$RGR = \frac{\ln \text{biomass at time 2} - \ln \text{biomass at time 1}}{\text{time 2 (days)} - \text{time 1 (days)}} \qquad (3.1)$$

The shoot and root samples were bulked across replicates of each treatment combination in order to collect enough sample to allow an analysis of total nitrogen to be completed. The bulked samples were ground using a Makla Mill, and analysed for total nitrogen using a LECO carbon/nitrogen analyser (LECO CN-2000). As the samples were bulked there were no replicates with which to carry out statistics.

Nodule scores were taken at each harvest using the following scoring system (from Peoples *et al.*, 1989):

0 = 0 nodules

1 < 5 nodules in the crown root zone

2 = 5-10 nodules in the crown root zone

3 > 10 nodules in the crown root zone

4 >10 nodules in the crown root zone and < 5 nodules below this zone

5> 10 nodules in the crown root zone and > 10 nodules below this zone.

Experimental data was analysed using analysis of variance. Although the data was not normal and analysis of variance was not the most appropriate analysis, it was decided to use this 'simple' test to give an indication of any treatment effects. More complicated analyses would include an extension of the Poisson technique which not only takes into account the score but the fact that there is a natural ordering to that measurement. After consulting a biometrician, it was decided that such detail was not required for this particular set of results.

3.3 RESULTS

3.3.1 Shoot biomass

There were significant interactions between the effects of herbicide application and harvest time ($p<0.001$; Figure 3.1) and those of nitrogen application rate and harvest time ($p<0.001$; Figure 3.2) on shoot biomass of chickpea. There was no significant interaction however, between the effects of herbicide application and nitrogen application rate ($p=0.918$) on chickpea shoot biomass. At the first 2 harvests, there was little difference in shoot biomass between herbicide sprayed and unsprayed plants (Figure 3.1). Flumetsulam reduced chickpea shoot biomass by 29% at the third

harvest (6 weeks) and 30% (9 weeks) at the fourth harvest in comparison to the unsprayed control (Figure 3.1).

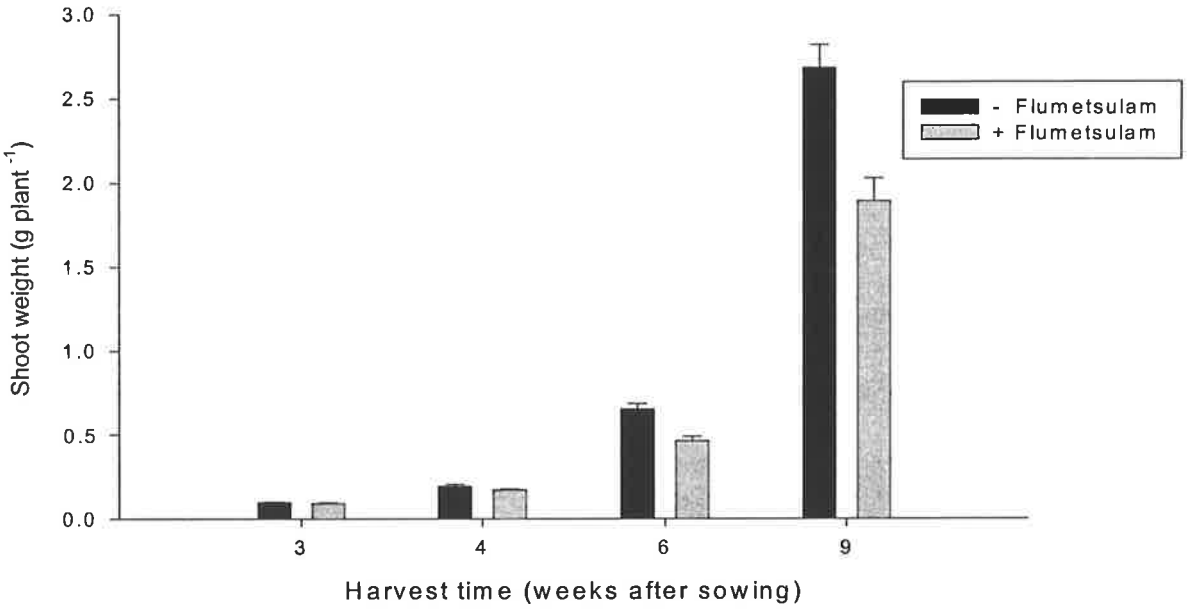


Figure 3.1: The effects of flumetsulam over four harvest times (3, 4, 6 and 9 weeks) on the shoot biomass of chickpeas grown in a glasshouse (averaged over all nitrogen treatments). Bars indicate standard error of mean.

Chickpea shoot biomass was affected by an interaction between nitrogen treatments and harvest time ($p<0.001$). At the first two harvests, there was little difference in shoot biomass between any of the nitrogen treatments (Figure 3.2). At the third harvest however, there was an increase in shoot biomass observed in the nitrogen treatments from 0 kg ha^{-1} to 20 kg ha^{-1} (Figure 3.2). The shoot biomass remained the same at 30 and 50 kg N ha^{-1}, then declined at the higher level of 100 kg N ha^{-1} (Figure 3.2). This trend was amplified at the fourth harvest (Figure 3.2). At the fourth harvest the maximum shoot biomass was attained at a nitrogen application rate of 50 kg N ha^{-1},

whilst the lowest shoot biomass was observed where no nitrogen fertiliser was applied (Figure 3.2).

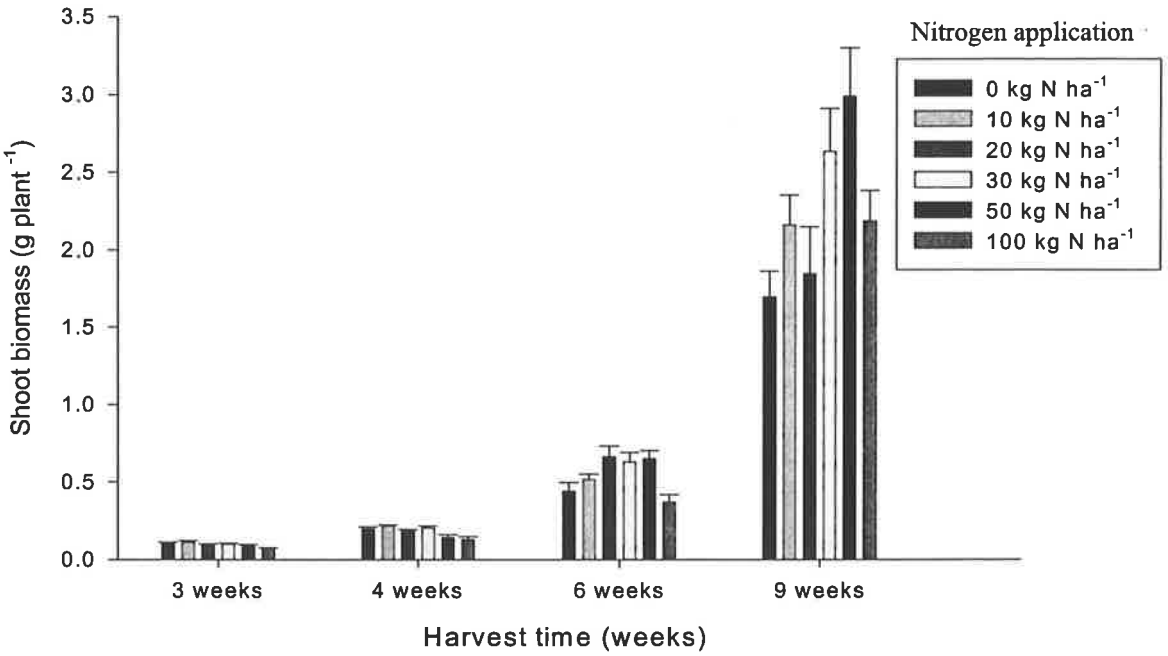


Figure 3.2: The effects of nitrogen fertiliser application (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) on shoot biomass of chickpeas, grown in a glasshouse, over time (averaged over both + and – flumetsulam treatments). Bars indicate standard error of mean.

3.3.2 Root biomass

Root biomass was significantly affected by an interaction between herbicide application and harvest time ($p<0.001$, Figure 3.3). There was no interaction between herbicide application and nitrogen application rate on root biomass ($p=0.604$). Root biomass was significantly affected by a main effect of nitrogen ($p<0.001$, Figure 3.4).

Chickpea root biomass had a similar trend to shoot biomass, with little difference between herbicide sprayed and unsprayed chickpea plants observed at the first two harvests (Figure 3.3). Flumetsulam application reduced root biomass by 24% and 34% at the third and fourth harvests respectively, compared to the untreated chickpea plants (Figure 3.3).

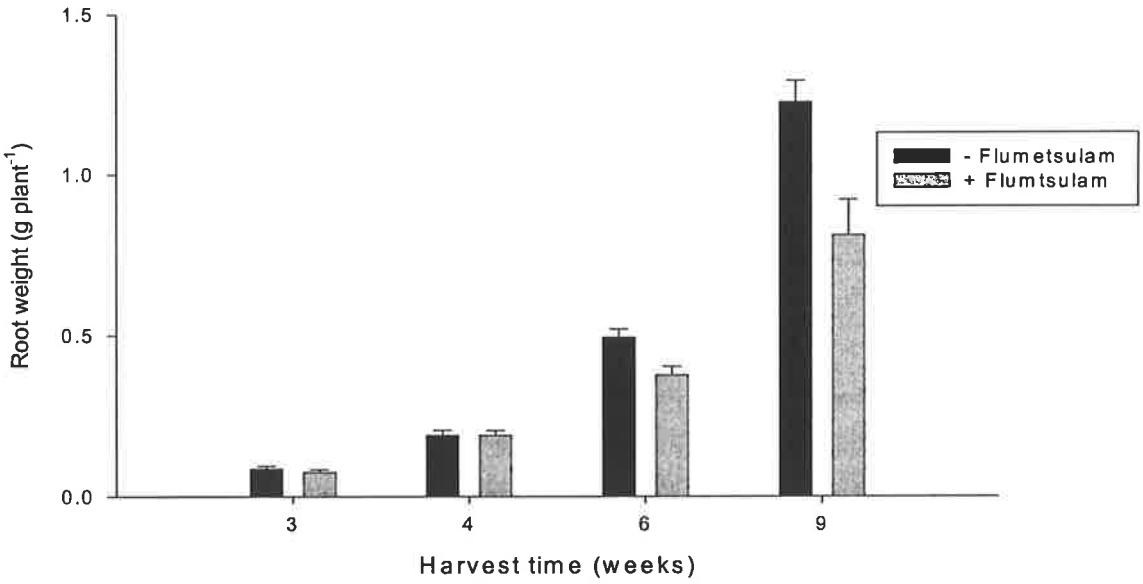


Figure 3.3: The effects of flumetsulam (presence (+) and absence (-)) on chickpea root biomass over time (3, 4, 6 and 9 weeks) (averaged over all nitrogen treatments). Bars indicate standard error of mean.

Root biomass was highest with nitrogen applications of 10 kg N ha^{-1}. The 0, 20, 30, and 50 kg N ha^{-1} nitrogen applications all showed similar root biomass, and the 100 kg N ha^{-1} application had the lowest root biomass (Figure 3.4).

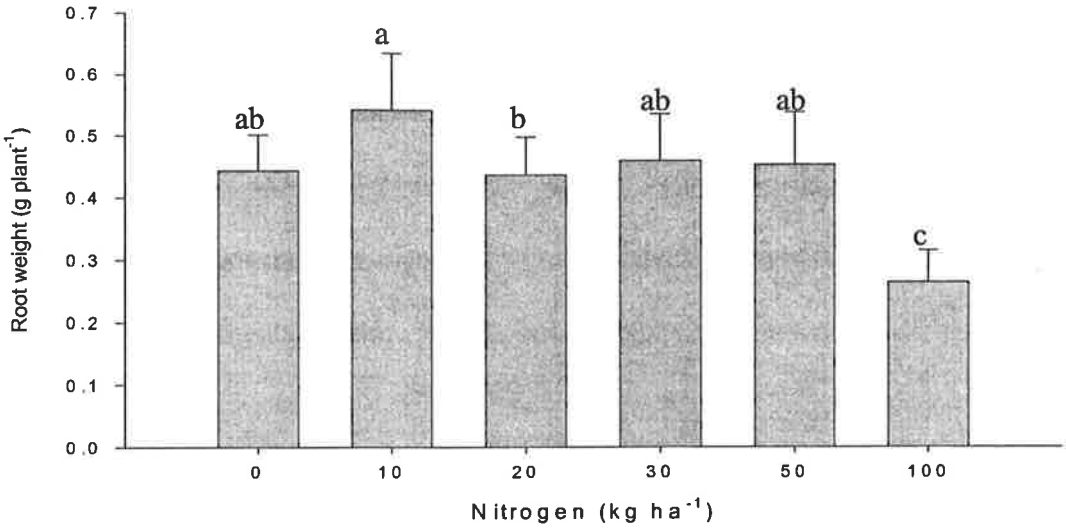


Figure 3.4: The effects of nitrogen fertiliser applications (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) on the root biomass of chickpeas (averaged over + and – flumetsulam treatments). Bars indicate standard error of mean.

3.3.3 Relative growth rates

Shoot and root weights were combined to calculate relative growth rates of the chickpea plants. The relative growth rates of chickpeas were significantly affected flumetsulam between the first and second harvests (p=0.007), second and third harvests (p=0.044), and third and fourth harvests (p=0.032). Table 3.2 shows the table of means for all relative growth rates of chickpea plants as affected by flumetsulam. Sprayed plants had a higher relative growth rate than unsprayed plants between the first and second harvests (Table 3.2). However, flumetsulam reduced the relative growth rates between the second and third, and third and fourth harvests (Table 3.2).

Table 3.2: Table of means of significant (α=0.05) flumetsulam effect on the relative growth rates of chickpea between the first and second, second and third and third and fourth samplings. Numbers in parentheses indicate standard error.

	Relative growth rate (g g^{-1} day^{-1})	
	Flumetsulam	
	-	+
Between harvest 1–2	0.102 (0.008)	0.122 (0.009)
Between harvest 2-3	0.077 (0.004)	0.059 (0.005)
Between harvest 3-4	0.060 (0.004)	0.053 (0.004)

The relative growth rate of chickpea plants between the second and third harvests was significantly affected by nitrogen fertiliser applications (Table 3.3). The highest relative growth rate was found with the addition of 50 kg N ha^{-1} and the lowest was with 0 kg N ha^{-1} (Table 3.3). Generally there was an increase in relative growth rate to the peak at 50 kg N ha^{-1} and then it declined with an application of 100 kg N ha^{-1} (Table 3.3).

Table 3.3: Table of means for nitrogen effects on relative growth rates of chickpeas between the second and third harvests. Numbers in parentheses indicate standard error of mean.

Nitrogen (kg N ha^{-1})	Relative growth rate (Harvests 2-3) ($g\ g^{-1}\ day^{-1}$)
0	0.056 (0.008)
10	0.057 (0.005)
20	0.073 (0.006)
30	0.069 (0.010)
50	0.087 (0.009)
100	0.066 (0.007)

3.3.4 Nitrogen content of chickpea shoots and roots

The nitrogen content data was not statistically analysed as the samples were bulked across replicates and there were no replicates for analysis. At the first and second harvest there was little difference in nitrogen content of chickpea shoots between different nitrogen treatments, and herbicide sprayed and unsprayed plants. At the third harvest, the highest nitrogen content of plants not treated with flumetsulam was noted for the 30 and 50 kg N ha^{-1} nitrogen treatments (Figure 3.5). The sprayed plants had nitrogen contents lower than unsprayed plants and the highest nitrogen content was noted for the 50 kg N ha^{-1} nitrogen treatment (Figure 3.5). The nitrogen content of chickpea shoots declined with the application of 100 kg N ha^{-1} nitrogen fertiliser in both the sprayed and unsprayed plants at the third harvest (Figure 3.5). Spraying chickpea

plants with flumetsulam reduced the nitrogen content of shoots at the fourth harvest (Figure 3.5). In both sprayed and unsprayed plants, at the fourth harvest, the maximum nitrogen content was observed following the application of 100 kg N ha^{-1} (Figure 3.5).

The nitrogen content of chickpea roots was similar across all nitrogen and herbicide treatments at the first harvest (Figure 3.6). At the second harvest, the 50 kg N ha^{-1} treatment in the unsprayed plants showed a higher nitrogen content than the other treatments, with little difference between other nitrogen and herbicide treatments (Figure 3.6). At the third harvest, the 0, 10 and 100 kg N ha^{-1} treatments in the unsprayed plants had higher contents, with all other treatments having a similar lower nitrogen content (Figure 3.6). At the fourth harvest, with the exception of the 10 kg N ha^{-1} treatment, the sprayed plants had lower root nitrogen contents than the unsprayed plants (Figure 3.6). The 0, 10 and 50 kg N ha^{-1} nitrogen fertiliser treatments in the unsprayed plants had the highest nitrogen content at the fourth harvest (Figure 3.6).

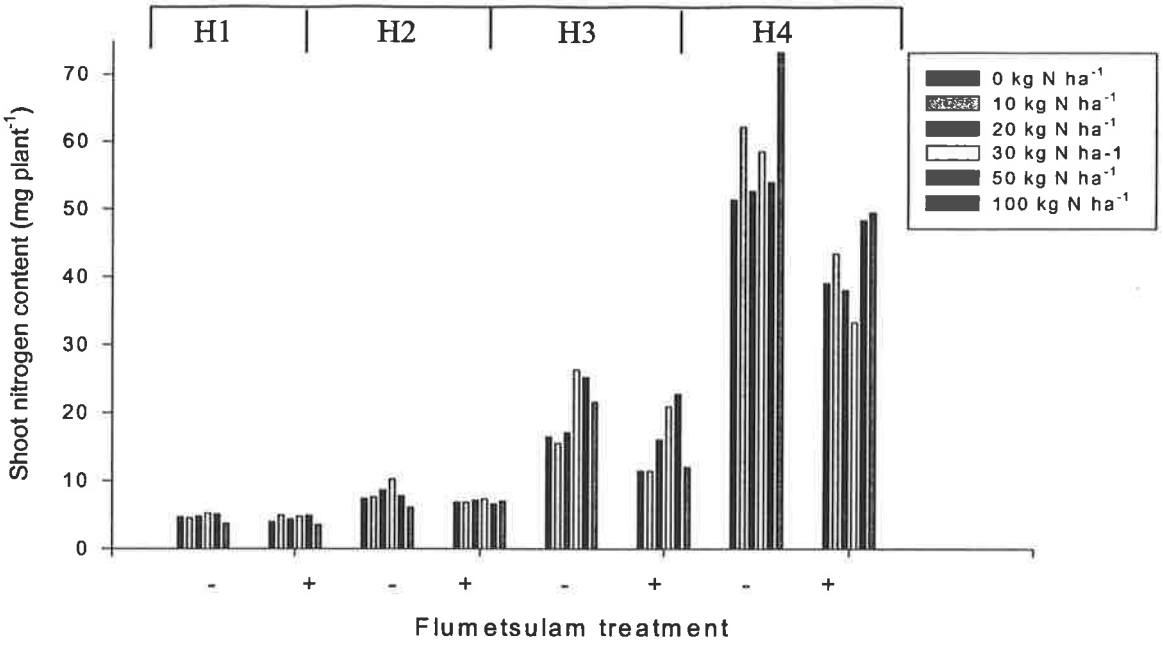


Figure 3.5: The effects of flumetsulam and nitrogen fertiliser application (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) on nitrogen content of chickpea shoots over time. -/+ indicate absence and presence of flumetsulam. H1, 2, 3, 4 indicate harvests at 3, 4, 6 and 9 weeks respectively.

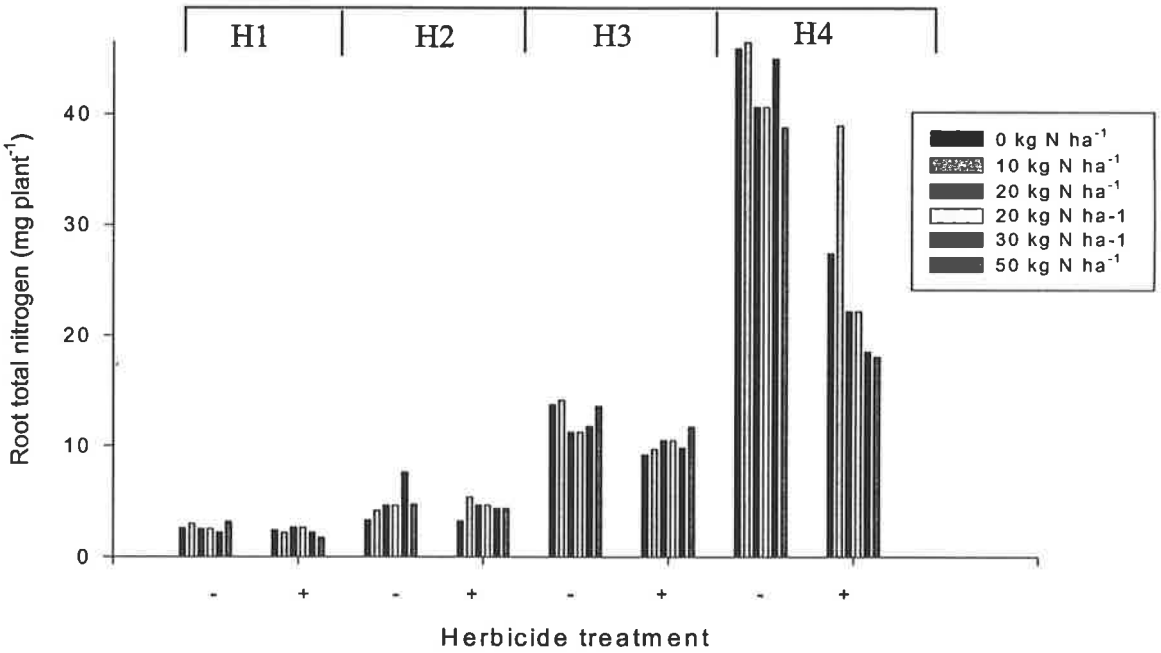


Figure 3.6: The effects of flumetsulam and nitrogen fertiliser (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) applications on nitrogen content of chickpea roots over time. -/+ indicate absence and presence of flumetsulam. H1, 2, 3, 4 indicate harvests at 3, 4, 6 and 9 weeks respectively.

Shoot % N of chickpea plants was reduced over time, but was not reduced by the presence of flumetsulam (Figure 3.7). At the first two harvests, shoot % N generally increased with increasing nitrogen fertiliser , however at the final harvest the 0 and 100 kg N ha^{-1} fertiliser applications showed the highest shoot % N (Figure 3.7).

Root % N was similar over the first three harvests with respect to each nitrogen fertiliser treatment (Figure 3.8). From the third to fourth harvests, root % N of chickpea plants treated with 0-30 kg N ha-1 increased (Figure 3.8). However, flumetsulam reduced the root % N of chickpea plants across all fertiliser treatments (Figure 3.8).

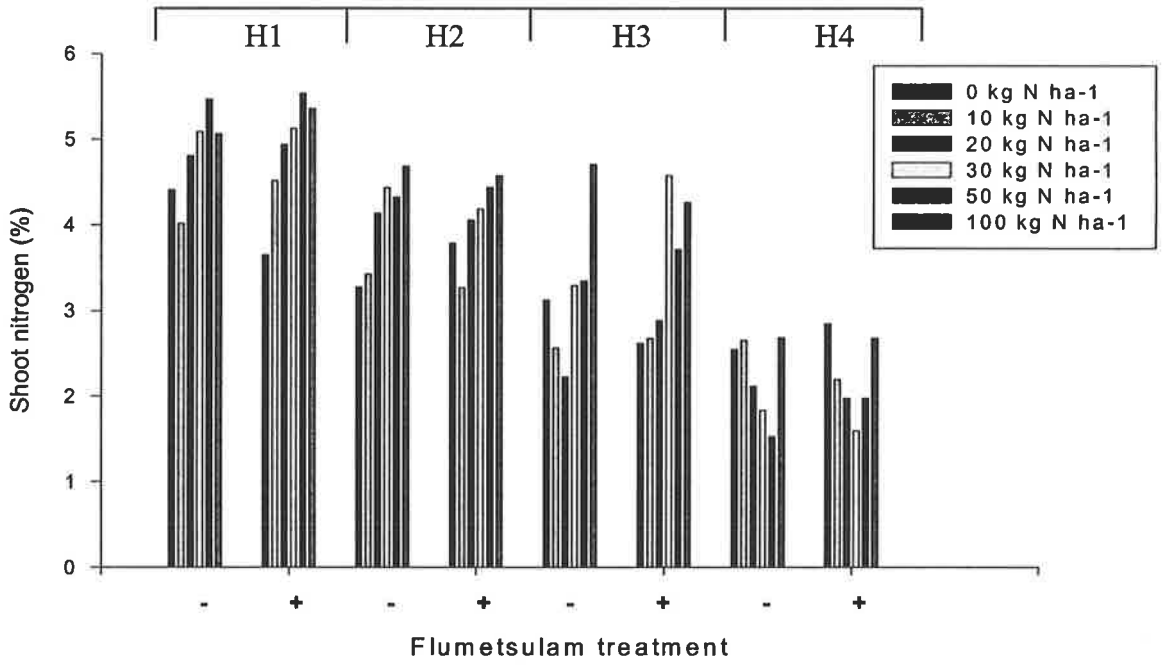


Figure 3.7: The effects of flumetsulam and nitrogen fertiliser (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) applications on % nitrogen of chickpea shoots over time. -/+ indicate absence and presence of flumetsulam. H1, 2, 3, 4 indicate harvests at 3, 4, 6 and 9 weeks respectively.

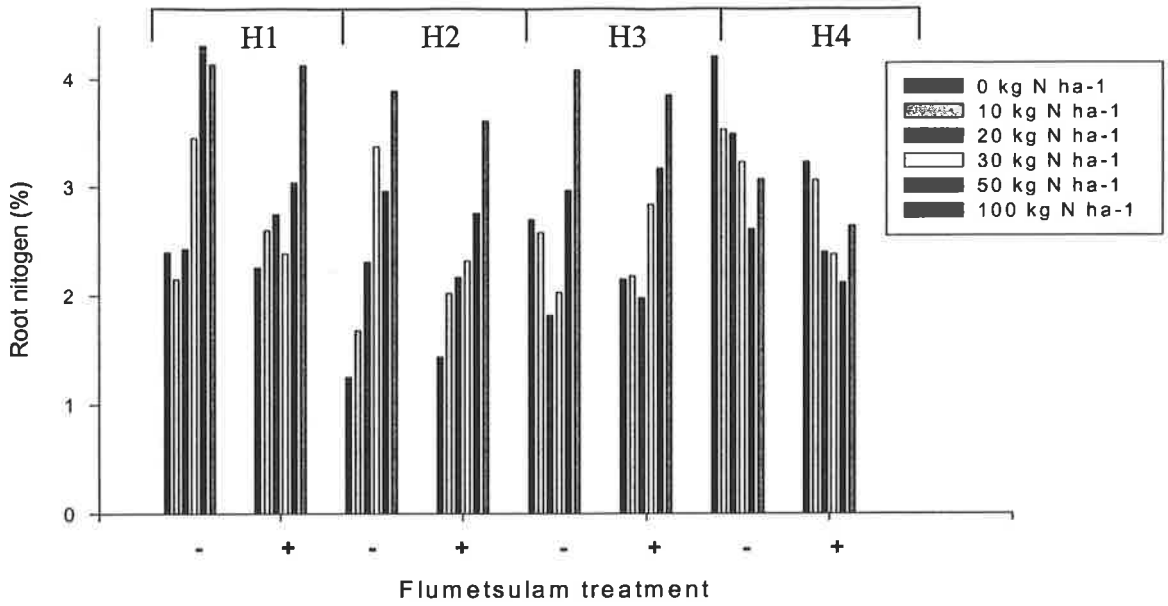


Figure 3.8: The effects of flumetsulam and nitrogen fertiliser (0, 10, 20, 30, 50, and 100 kg N ha^{-1}) applications on % nitrogen of chickpea roots over time. -/+ indicate absence and presence of flumetsulam. H1, 2, 3, 4 indicate harvests at 3, 4, 6 and 9 weeks respectively.

3.3.5 Nodule scores

There was a three way interaction between herbicide application, nitrogen application rate and harvest time ($p<0.001$) on the nodule scores for the chickpea plants. There was little difference in the nodule scores of sprayed and unsprayed plants (Figure 3.9). The number of nodules decreased with the 50 and 100 kg N ha^{-1} nitrogen applications, although the lower levels of nitrogen had little effect (Figure 3.9). The number of nodules was highest at the third and fourth harvests with all treatments except 50 and 100 kg N ha^{-1} with nodule scores of between 4 and 5 (Figure 3.9).

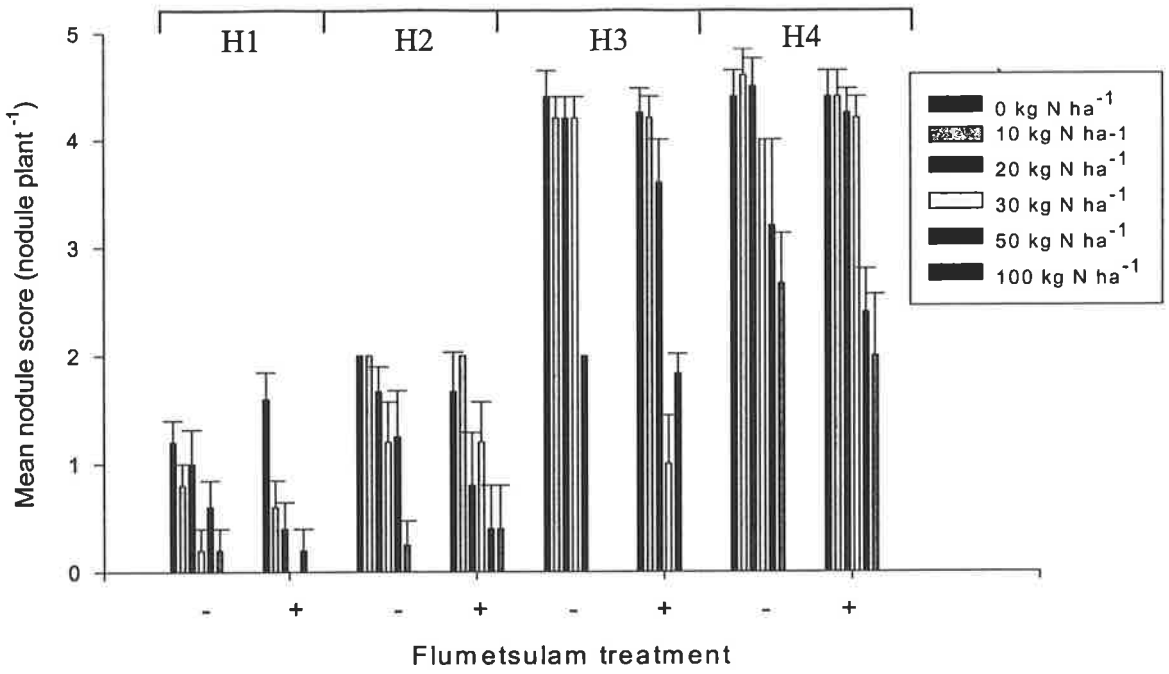


Figure 3.9: Mean nodule scores for chickpea plants over time following applications of flumetsulam and nitrogen (0, 10, 20, 30, 50, 100 kg N ha^{-1}). -/+ represent absence and presence of flumetsulam. H 1, 2, 3, 4 represent harvests at 3, 4, 6 and 9 weeks respectively. Bars indicate standard error of mean.

3.4 DISCUSSION

Flumetsulam, an ALS-inhibiting herbicide, acts by inhibiting cell division and growth (Brown, 1990; Ray, 1982a) and as such it can be expected that growth rates would slow down in sensitive species after herbicide application. Three weeks after spraying flumetsulam at the recommended application rate, root and shoot biomass of chickpea plants was reduced, suggesting that chickpeas may be sensitive to the herbicide. The reduction in biomass increased further six weeks after spraying. Shoot biomass responded positively to nitrogen fertiliser applications. At two weeks, flumetsulam treated plants had higher relative growth rates than those that were not sprayed. However this changed as the experiment continued, with flumetsulam reducing the relative growth rates of chickpeas, indicating that flumetsulam was inhibiting the ability of the plants to produce more biomass. Although growth rates and

the production of biomass were initially unaffected by flumetsulam, after six weeks flumetsulam treated plants were producing less biomass than the untreated plants, indicating that flumetsulam inhibited the growth of chickpeas.

As with chickpea shoot biomass, growth rates responded positively to the addition of nitrogen fertiliser. Mckenzie *et al* (1992) also observed that nitrogen application at 6 rates from 0 –100kg N ha^{-1} increased shoot dry weights per plant by 36 – 42%. It should be noted that in the current experiment the application of 100kg N ha^{-1} had lower biomass and relative growth rates than other nitrogen fertiliser treatments. This reduction was attributed to the treatment not mixing throughout the pot, leading to an observed concentration of salts at the surface and the shoots appearing stunted and 'burnt'.

Under normal circumstances (ie in the absence of herbicide) plant biomass and nodulation would increase over time, and high levels of nitrogen would inhibit nodulation and lower nodule scores. The reduction in nodule formation due to the presence of inorganic nitrogen was consistent with other studies where nodulation of chickpeas was found to decrease with increasing levels of nitrogen (Calcagno *et al.*, 1989; Jessop *et al.*, 1984). The only reduction in chickpea nodule scores due to flumetsulam application were observed in the third and fourth harvests where high (20 – 100 kg N ha^{-1}) nitrogen fertiliser rates were applied, suggesting that high nitrogen levels were more responsible for the reduction than the herbicide application. The reduction in nodule score by 6 – 9 weeks is also consistent with the observed reduction in chickpea dry matter at this time and may reflect the reduction in available sites for nodulation. The number of nodules did increase between the third and fourth harvests at the higher rates of nitrogen fertiliser (50 and 100 kg N ha^{-1}), but the difference between plants

sprayed and not sprayed with flumetsulam also increased. The effects of flumetsulam were more obvious by the six week harvest when maximum nodulation was observed. The high nodule scores of the plants treated with low levels of nitrogen fertiliser in this study, were representative of excellent nodulation and the chickpea plants would have had an excellent potential for nitrogen fixation (Peoples *et al.*, 1989).

Although there were no differences in nodule scores between chickpea plants sprayed with flumetsulam and those that were not sprayed, there was still a reduction in the nitrogen content of both roots and shoots, six weeks after spraying. The reduction in shoot nitrogen content is a reflection of the reduction in shoot biomass, as shoot % N was not reduced by flumetsulam. However, there was a reduction in root % N at the final harvest and therefore nitrogen content was reduced by flumetsulam and was not simply a reflection of the reduction in root biomass. Plant nitrogen content, particularly in the roots and with flumetsulam application, decreased with the addition of high levels of nitrogen. This reduction in nitrogen content was a reflection of reductions in both root biomass and % root N. Reduced root biomass would lead to less ability to take up nitrogen from the soil.

The results of this pot experiment differ from studies of flumetsulam on pasture legumes in the field. Ewers and Phillips (1993) found that yields of six pasture legume species were unaffected by flumetsulam. Flumetsulam was found to give good weed control and high seed yields when applied to medic pastures in South Australia (Dickinson *et al.*, 1993). These differences may be due to chickpeas being more sensitive to flumetsulam than medic and other pasture legumes. Flumetsulam may become less available to plants in the field due to adsorption to soil (Leake 1991; Fryer and Makepeace, 1977).

It is important to note that several important differences exist between glasshouse or pot trials and field experiments. In pot trials, plant roots are confined to a smaller volume of soil compared to plants growing in the field, and therefore the soil volume to root ratio is lower. In addition, field and pot trials have differing root, nutrient and herbicide profiles that alter the pattern of absorption and uptake (Jettner *et al.*, 1999). In undrained pot trials, such as the one discussed in this chapter, nutrients, herbicide and roots are contained in a small volume (Osbourne *et al.*, 1993) and all roots would have greater exposure to the herbicide. However, in field trials it is possible for plant roots to extend below the level of herbicide contamination. A study investigating the effects of heavy metals on lettuce and onions found that glasshouse trials gave much higher concentrations of heavy metals in plant biomass than field trials (de Vries and Tiller, 1978). The higher concentrations of heavy metals in lettuce and onion found in the glasshouse trial was unlikely to be due to different rooting patterns (i.e. penetration into untreated soil in the field) because of the shallow rooting system of the plants studied (deVries and Tiller, 1978). de Vries and Tiller (1978), suggest that the different results from the glasshouse trial compared to the field trial, may have been due to differences in soil and air temperatures, air humidity and movement, soil moisture status and the quality of incident light. As a result, care should be taken with extrapolating results of pot and glasshouse experiments to the field due to differences in environmental conditions, size and type of pots used and growing conditions (de Vries, 1980). The results collected in this experiment should therefore be interpreted cautiously, as the herbicide effects on the plant may be overestimated.

3.5 SUMMARY AND KEY FINDINGS

The results of the study discussed in this chapter have shown that spraying flumetsulam at the recommended application rate reduced the total biomass, growth rate and nitrogen content of chickpeas grown in pots in a glasshouse. The reduction in nitrogen content of chickpea shoots was a reflection of the impact on shoot biomass, but % N of chickpea roots was reduced by flumetsulam, suggesting that the herbicide may be directly reducing nitrogen content of the chickpea to some extent. The addition of nitrogen fertiliser did not improve either the growth or nitrogen content of flumetsulam treated chickpeas, suggesting that the plant can not fertilise its way out of the problems and the herbicide will cause problems for chickpeas in the field.. This does not necessarily imply that the nitrogen fixing capabilities of the plant are not affected and may simply be a reflection of the reduction in root biomass, resulting in less nitrogen fertiliser uptake from the soil. This suggests that caution may be required when using flumetsulam on chickpea crops in the field. However, care should be taken when interpreting results of pot trials and applying them to field situations. The next two chapters will investigate the effects of flumetsulam and the residues of sulfonylureas on the growth and yield of medic and chickpea in the field.

CHAPTER 4

THE EFFECTS OF ALS-INHIBITING HERBICIDES ON GROWTH AND PRODUCTION OF *MEDICAGO RUGOSA*

4.1 INTRODUCTION

Rainfed or dryland farming systems of Australia involve rotations of crops, pastures and fallows (Squires, 1991). In South Australia wheat is a major crop, with the 1,534,900 ha sown in 1996 yielding 2,794,900 tonnes (ABS, 1996 -1997). Crop selective sulfonylureas have been commercialised for use in wheat, barley and rice (Brown, 1990) and any negative impact of the selective herbicides on these crops would be expected to be minimal. Chlorsulfuron (Glean®) is registered for post-sowing use in wheat, barley, oats, triticale and cereal rye in all states of Australia (Chambers, 1995). Triasulfuron (Logran®) is registered for use in wheat in all Australian states except Tasmania (Chambers, 1995). Sulfonylureas are widely used to control broadleaf weeds and during 1993 chlorsulfuron and triasulfuron were applied to an estimated 760,000 ha of alkaline cropping soils in Australia (Stork, 1995).

Crop rotations are used to break the life cycles of both pests and weeds, maintain soil fertility, and prevent soil erosion (Tow and Schultz, 1991). Pasture or grain legume rotations are common farming practice in South Australia, and residual nitrogen from these rotations has a significant beneficial impact on the subsequent cereal yields (Tow & Schultz, 1991).

Medicago spp. are often the dominant pasture species in dryland farming regions of southern Australia (Unkovich *et al.*, 1997) and are important for nitrogen fixation and

livestock feed (Carter, 1987). *Medicago truncatula* was found to fix approximately 90 kg N ha^{-1} (Peoples *et al.*, 1995b). In southern Australia, in 1992, 11,100,000 ha were under clover and/or medic pasture (ABS, 1993). The productivity of these pastures has declined over the last 10 to 15 years (Carter, 1987). A reduction in lucerne (*M. sativa*) productivity was found to be related to a reduction in effective *Rhizobium* in the soil following soil acidification and herbicide use (Koopman *et al.*, 1995; Koopman *et al.*, 1993).

Pasture production, forage utilisation and animal production can be improved by managing weeds in pastures (Smith and Martin, 1995). Weed control in pastures can be achieved by mechanical (e.g. tillage, mowing) or cultural methods (e.g. crop rotation, burning, competition, grazing) as well as by biological weed management or chemical weed management (e.g. herbicides) (Smith and Martin, 1995). Flumetsulam (Broadstrike®) is registered for use in medic and clover seed crops and pastures in South Australia (Chambers, 1995). Flumetsulam has reduced numbers of the weed doublegee (*Emex australis*) by up to 98% and increased the seed set of medic (Gilmour, 1996).

Although some herbicides may improve the productivity of pastures through a reduction in weeds, others may be deleterious. A reduction in medic shoot biomass was observed, following application of sulfonylurea herbicides to cereal crops in previous years (Gillett & Holloway, 1996; Evans *et al.*, 1993; Rovira *et al.*, 1993).

The pot trial discussed in Chapter 3 found that shoot and root biomass of chickpea plants were reduced by flumetsulam. Plant roots in pot experiments are more constrained than those grown in the field and are likely to access more herbicide. Because of this, herbicide effects may be more pronounced in pot experiments. The

field trial discussed in this chapter investigated the effects on medic because of its importance in agricultural systems. A second field trial investigated the effects on chickpeas and will be discussed in Chapter 5. In addition to flumetsulam, the residual effects on medic from selected sulfonylurea herbicides applied to cereal crops were also investigated, as a pasture rotation may be exposed to residues of these herbicides. It is possible that medic plants sprayed with flumetsulam will also come into contact with sulfonylurea residues from previous crop applications and because of this the combined effects of these herbicides will also be investigated.

The objectives of this field experiment were to:

1. Determine the effects of chlorsulfuron and triasulfuron on the biomass and yield of wheat (*Triticum aestivum*);

2. Quantify the residual effects of chlorsulfuron and triasulfuron applied to preceding wheat crops on the growth and seed yield of *M. rugosa*;

3. Determine the 'direct' (or 'in-crop') effects of applying flumetsulam to *M. rugosa* pastures on the growth and seed yield of *M. rugosa*;

4. Measure the cumulative or combined effects of these herbicides (i.e. sulfonylurea residues remaining from the previous growing season and in-crop usage of flumetsulam) on growth and seed yield of *M. rugosa*.

In this and the following chapter, residual effects are defined as those observed in a season other than the one of application, whereas direct or in-crop effects are those that were observed in the year of application. Cumulative effects are the combination of effects from residues remaining from a previous application and those observed during the season of application.

The experiment was conducted over two years. In the first year, chlorsulfuron and triasulfuron were applied and wheat was sown following common agronomic practice, in order to investigate the residual effects on medic the following season. During the second year, medic was sown and flumetsulam was applied with the objective of investigating direct, residual and cumulative effects of these herbicides.

4.2 MATERIALS AND METHODS

4.2.1 Site selection and soil characteristics

The field trial was established on a Sodic, Supracalcic Red Chromosol soil (Soil Taxonomy (USDA, 1994): Natrixeralf) at the Roseworthy Campus of The University of Adelaide. The soil characteristics of the site are summarised in Table 4.1. The main criteria used to select the experimental site were: (i) pH range of 8.0 – 8.5 (H_20) due to sulfonylurea persistence under alkaline conditions, and (ii) no history of sulfonylurea usage in the previous seven years to ensure that any effects observed are the result of the experimental treatments only.

Table 4.1: Soil characteristics for paddock West 10 at the Roseworthy campus of the University of Adelaide.

Soil characteristic	Value
pH (water 1:5)	8.3
pH (calcium chloride)	7.7
Organic carbon	1.21%
Extractable phosphorous	22 mg kg^{-1}
Extractable potassium	405 mg kg^{-1}
Soil salinity EC (1:5)	0.15 dS m^{-1}
Soil Salinity Ece (est)	1.5 ds m^{-1}
Free lime	Moderate
Soil texture	Clay loam
Cation exchange capacity	28.03 mequiv $100g^{-1}$

4.2.2 Treatments

Prior to the establishment of field treatments the site was sprayed with glyphosate to remove existing weeds. In the first year of the experiment (1996), chlorsulfuron {2-chloro-*N*–[[(4-methoxy–6–methyl–1,3,5–triazin-2-yl)amino]carbonyl] benzenesulfonamide}, and triasulfuron {1-[2-(2-chloromethoxy)phenylsulfonyl]-3-(4-methoxy-6-methyl-1,3,5-triazin-2-yl)} were applied to the appropriate plots at two application rates (at the recommended and double the recommended field application rate) (Table 4.2). Double the recommended field application rates for chlorsulfuron and triasulfuron will be referred to throughout the chapter as chlorsulfuron x 2 and triasulfuron x 2 respectively. Trifluralin [2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl) benzenamine] was included in the experiment as a non ALS-inhibiting herbicide and was applied at the recommended application rate and incorporated into the soil according to the label recommendation (Table 4.2). In addition, a nil treatment where no herbicide was applied, was included. The actual herbicide application rates are presented in Table 4.2. All herbicides were applied pre-sowing on a fine, mild day with

a northeast breeze of 4km h^{-1} using a 10 m boom. The wheat was sown four days after spraying.

In the second year of the experiment (1997), naturally regenerating *Medicago rugosa* cv Paraponto Desr (Oram, 1990) (600 – 700 plants m^{-2}) was the experimental plant. Flumetsulam (*N*-(2,6-difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-*a*]pyrimidine-2-sulfonamide) was sprayed on the morning of 14th July 1997 at the three trifoliate leaf stage as per label recommendations, over the top of the residual sulfonylureas that were applied in 1996. The application rate of flumetsulam is shown in Table 4.2. Weather conditions during flumetsulam application were mild, with slight winds overcast skies and a forecast for possible rain later in the evening.

Targa (94 g l^{-1} quizalofrop-p-ethyl) was sprayed (350 ml ha^{-1}) over the whole site later in the second season (22nd of September) to control grass weeds.

Table 4.2.: Active ingredients and rates of herbicides used in 1996 and 1997.

Herbicide	Active Ingredient	Rate (g ai ha^{-1})
Glean	Chlorsulfuron	15
Glean x 2	Chlorsulfuron	30
Logran	Triasulfuron	26.25
Logran x 2	Triasulfuron	52.5
Treflan	Trifluralin	1 L ai ha^{-1}
Broadstrike	Flumetsulam	25

4.2.3 Experimental design

In the first year of this experiment, a randomised block design with three replicates was used (Figure 4.1). In the second year, the experiment was modified to a strip plot design and flumetsulam was applied over the pre-existing sulfonylurea treatments (Figure 4.2).

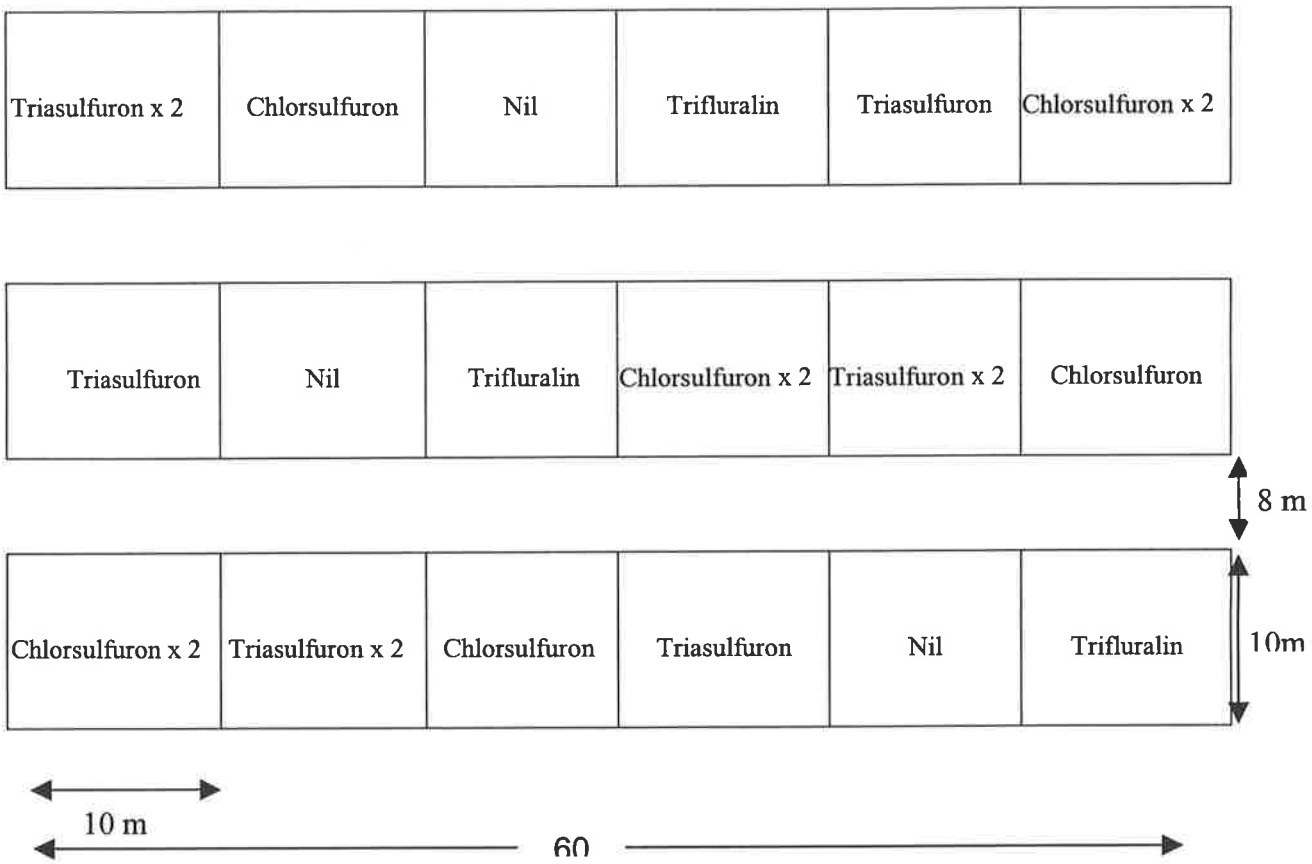


Figure 4.1: Field plan for experiment investigating effects of ALS-inhibiting herbicides on *M. rugosa*. Year one: wheat. See Table 4.2 for herbicide application rates.

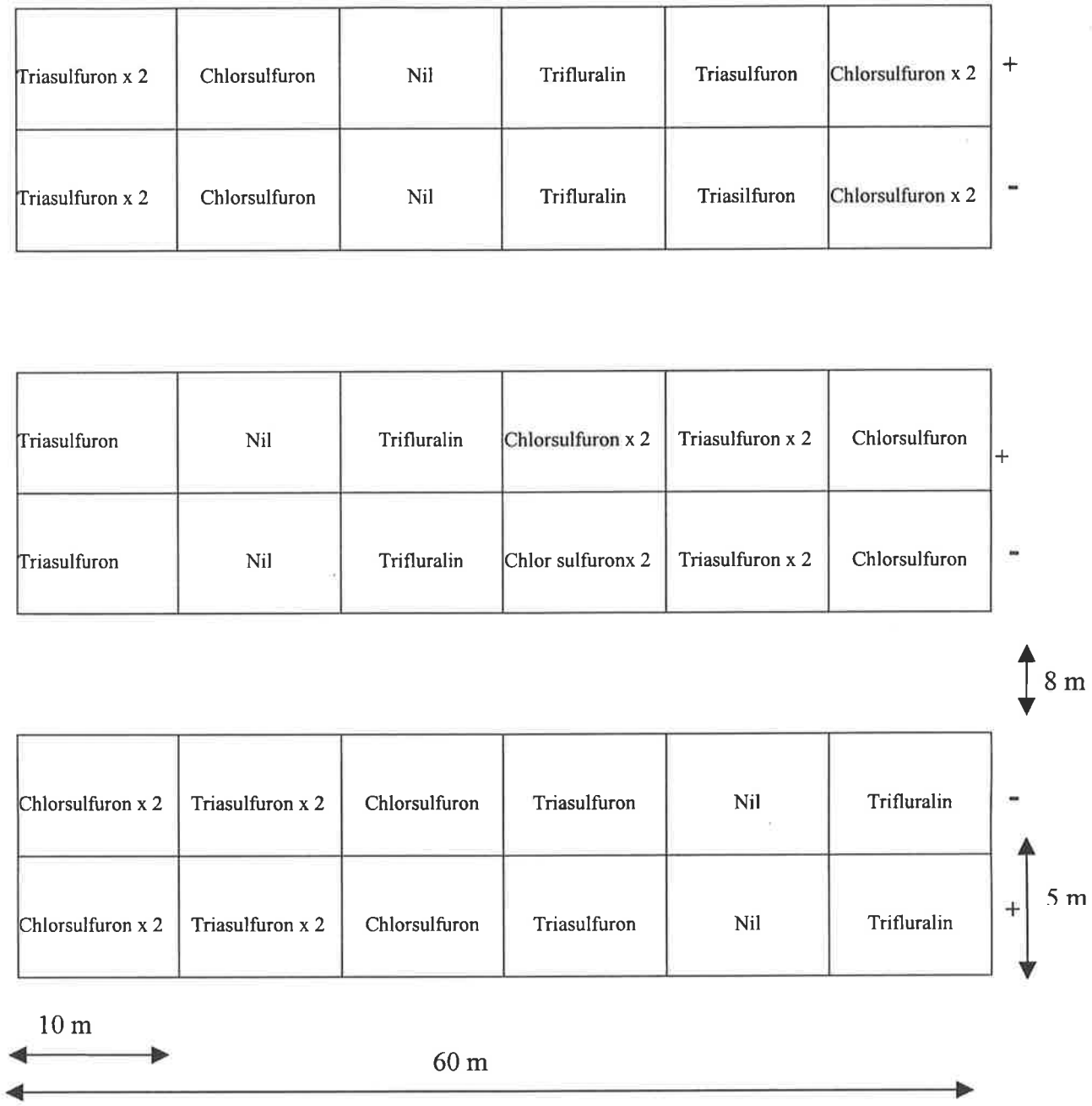


Figure 4.2: Field outline for year 2 with *M. rugosa*. (+ and – refer to with and without flumetsulam respectively. See Table 4.2. for application rates of herbicide).

4.2.4 Sampling and data collection

Wheat biomass cuts were taken at anthesis in October 1996 and the samples consisting of two quadrats measuring 40 cm x 40 cm were taken from each plot. The wheat was harvested in December 1996 using a Kew plot harvester, and biomass cuts were dried at 60°C and the dry weights were recorded.

Table 4.3 summarises the sampling strategy for the second year when medic was grown. The shoot samples from both years were dried at 60°C to determine the dry weights. This data was used to calculate relative growth rates (RGR) using the equation 4.1:

$$RGR = \frac{\ln(\text{biomass at time 2}) - \ln(\text{biomass at time 1})}{\text{time 2 (days)} - \text{time 1 (days)}} \qquad (4.1)$$

Shoot samples from flowering (peak biomass) were analysed for macro and micro nutrients and total nitrogen. The nutrient analysis was undertaken to ensure that nutrient concentrations in the shoots were within adequate guidelines for medic plants and to investigate whether the herbicides in question impacted on the nutrient concentrations. Nutrient concentrations were measured by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) analysis of samples that were ground using a Makla Mill (< 2 mm), and digested with nitric acid (Zarcinas and Cartwright, 1987). The results were compared to adequate nutrient concentrations for another medic species, *M. truncatula* (Reuter and Robinson, 1997), because comparative data was not available for *M. rugosa*.

Nitrogen concentrations were determined using a LECO carbon/nitrogen analyser (LECO CN-2000) and samples were ground using a Makla Mill (< 2 mm).

Data from biomass cuts and yield were analysed using analysis of variance (significant @ $\alpha = 0.05$) that was modified for strip plot design with the program Genstat 5 Release 4.1 (Payne, 1993). The treatment effects included in the analysis of variance were residues, flumetsulam, sampling time and their interactions. For results from the second year, residue effects refer to effects from residues of the 1996 herbicide application (i.e. chlorsulfuron, chlorsulfuron x 2, triasulfuron, triasulfuron x 2 and trifluralin). Flumetsulam effects refer to effects of flumetsulam applied in 1997 and are thus absent from the analysis completed for 1996. Where the analysis of variance identified significant treatment effects, Tukey's H.S.D. test was performed to identify which components of each treatment differed significantly. Graphs of significant treatment effects and interactions show means obtained from an average of all other treatments.

Table 4.3: Sampling times and methods of collection for field trial.

	Date	Time	Method	Samples taken
1	08/07/97	Pre – Broadstrike application	5 cores per plot (7.5 cm diameter, 10 cm deep)	Shoots (bulked by plot)
2	25/07/97	Post – Broadstrike (2 weeks)	3 quadrats per plot (20 x 40 cm)	Shoot biomass
3	09/09/97	Flowering	3 quadrats	Shoots
4	02/10/97	Late flowering	3 quadrats	Shoots
5	03/12/97	Harvest	2 quadrats (1m x 0.5 m)	Seed yield

4.3 RESULTS

4.3.1 Biomass and yield for wheat (year one)

The biomass of wheat at anthesis did not differ across the herbicide treatments ($p=0.906$) and the wheat yield in 1996 was not significantly affected by any of the herbicides ($p=0.583$).

4.3.2 Shoot biomass of *M. rugosa* from second year of experiment.

The shoot biomass of medic was not affected by an interaction between herbicide residues from the previous year, flumetsulam application and sampling time (Table 4.4). Additionally, medic shoot biomass was not affected by an interaction between flumetsulam and sampling time or flumetsulam and herbicide residues (Table 4.4).

The shoot biomass of medic was affected by an interaction between herbicide residues from the previous year and sampling time (Table 4.4). The shoot biomass of medic increased over time (Figure 4.3). At the final sampling, the nil herbicide treatment showed the highest biomass (Figure 4.3). Chlorsulfuron, chlorsulfuron x 2, triasulfuron, triasulfuron x 2 and trifluralin reduced the biomass of medic plants by 37%, 61%, 39%, 41% and 22% respectively when compared to the plants from the nil herbicide treatment (Figure 4.3). Chlorsulfuron x 2 had the lowest shoot biomass (Figure 4.3).

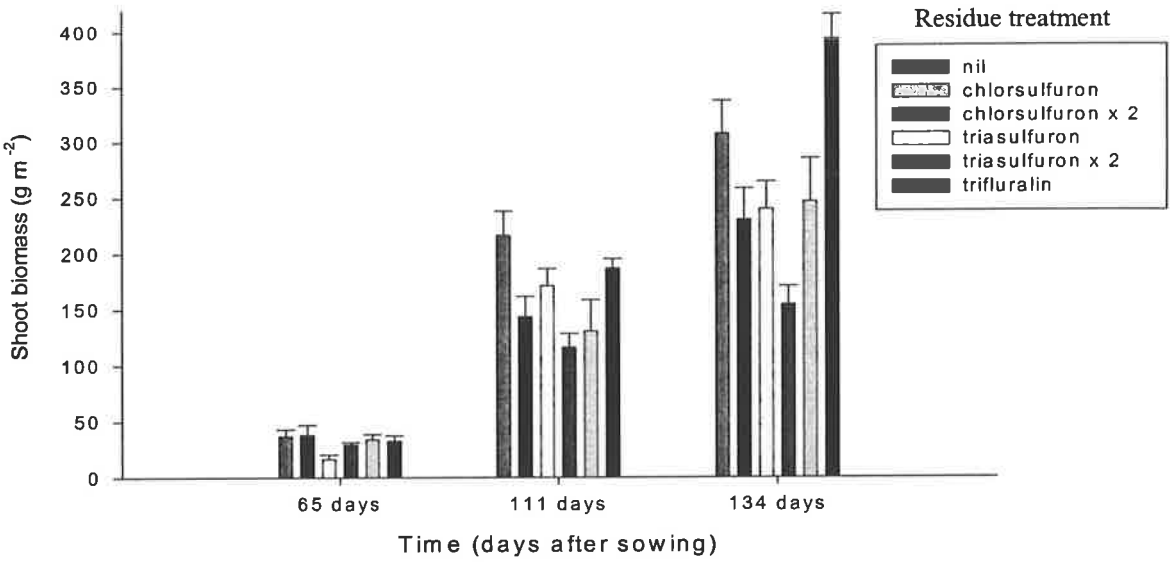


Figure 4.3: The effects of trifluralin and ALS-inhibiting herbicide residues from the previous year and sampling time on shoot biomass of *M. rugosa*. Bars indicate standard error of mean.

4.3.3 Relative growth rate of medic

The relative growth rate of medic was not affected by the interaction between herbicide residues and flumetsulam application (Table 4.4). However, the relative growth rate of medic between the third and fourth sampling was significantly affected by herbicide residues ($p=0.048$). Medic plants treated with double the recommended application rate of chlorsulfuron, had a relative growth rate that was 61% lower than the nil herbicide residue treatment (Figure 4.4).

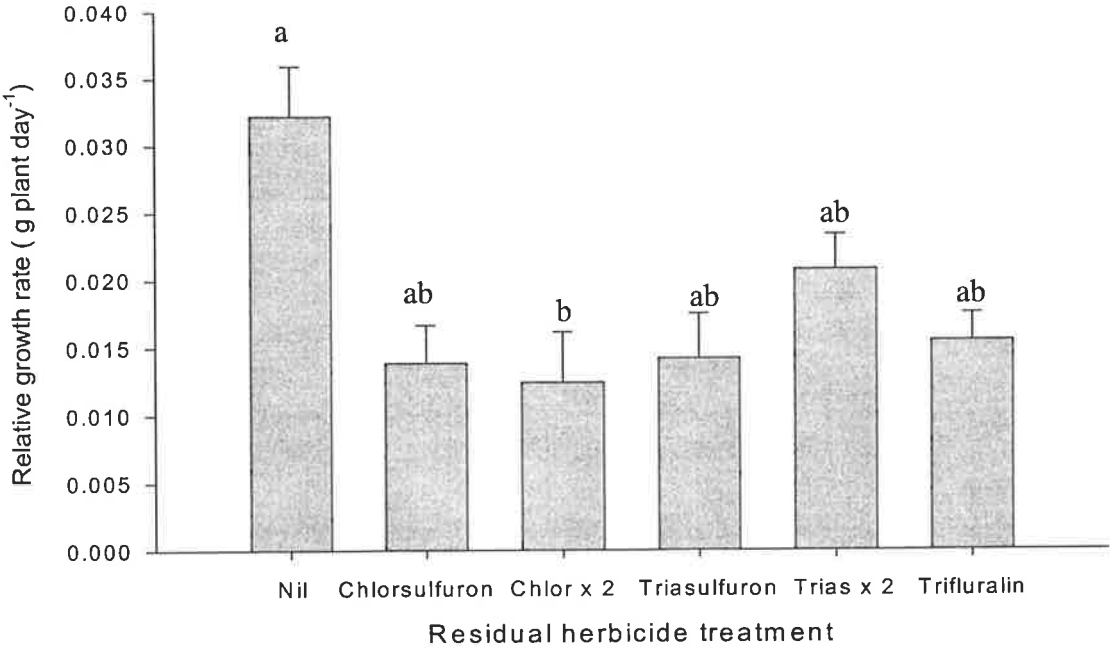


Figure 4.4: Effects of trifluralin and ALS-inhibiting herbicide residues from the previous year on relative growth rate of *M. rugosa* shoots between flowering and early senescence. Chlor x 2 and Triasulfuron x 2 indicate chlorsulfuron and triasulfuron respectively at double the recommended application rate. Bars indicate standard error of mean; letters indicate significantly similar values as determined by Tukey's H.S.D. test.

Table 4.4: Summary of analysis of variance results for effects of sulfonylurea herbicide residues and flumetsulam on shoot biomass, relative growth rates and seed yield of *M. rugosa*. Significant (α=0.05) effects are shown in bold.

Variable	Source	P-value
Shoot Biomass	Residue	**0.009**
	Time	**<0.001**
	Flumetsulam	0.828
	Residue*time	**<0.001**
	Flumetsulam*time	0.788
	Residue*Flumetsulam	0.218
	Residue*Flumetsulam*time	0.795
Relative growth rate (sampling 1 - 2)	Residue	0.065
	Flumetsulam	0.443
	Residue*Flumetsulam	0.576
Relative growth rate (sampling 2 - 3)	Residue	0.087
	Flumetsulam	0.725
	Residue*Flumetsulam	0.474
Relative growth rate (sampling 3 - 4)	Residue	**0.048**
	Flumetsulam	0.561
	Residue*Flumetsulam	0.110
Yield	Residue	0.100
	Flumetsulam	0.655
	Residue*Flumetsulam	0.391

4.3.4 Seed Yield

There were no significant interaction or main effects of herbicide residues or flumetsulam application on seed yield of medic (Table 4.4).

4.3.5 Total nitrogen

Total nitrogen and % N of medic shoots was not affected by any interaction, flumetsulam or herbicide residue effects (Table 4.5).

Table 4.5: Summary of analysis of variance results for effects of herbicides on Total nitrogen of *M. rugosa* shoots (α=0.05).

Variable	Source of variation	P –value
Total Nitrogen	Residue	0.088
	Flumetsulam	0.790
	Residue*flumetsulam	0.294
% N	Residue	0.088
	Flumetsulam	0.790
	Residue*flumetsulam	0.294

4.3.6 Nutrient Analysis

Nutrient analyses were performed to ensure that the only effects observed were due to herbicide effects and not limiting nutrients. The data obtained were compared to values for *M. truncatula* (Reuter and Robinson, 1997). The majority of nutrients were above adequate levels for *M. truncatula* (Table 4.6). The exceptions were zinc and manganese and although these were below adequate levels, the levels were consistent across all treatments (Table 4.6).

Table 4.6: Major nutrient analysis of *M. rugosa* shoots at flowering showing herbicide residue and flumetsulam treatments. Adequate , marginal (marg.) or critical deficiencies (cd) of a similar species, *M. truncatula* are shown in bold for each nutrient. Standard deviations of results are shown in parentheses. Results of analysis of variance are shown with significant results shown in bold.

Residue	Flumetsulam	P 2600-4500	K 11000-18000	S c.d <1600	Ca 8000-12000	Mg 2000-5500	Cu >5	Zn 17 marg.	Mn 35-250	B 25-50
Nil	-	3100 (100)	29666.7 (4509.2)	2066.7 (115.5)	19266.7 (1750.2)	3100 (173.2)	5.9 (0.2)	18.07 (3.2)	26.6 (2.0)	54 (5.1)
Nil	+	2967.7 (152.8)	30000 (4000)	1673.3 (196.6)	19000 (1757.8)	3066.7 (152.8)	5.5 (0.4)	14.1 (0.9)	24.3 (1.4)	54.4 (3.6)
Chlorsulfuron	-	3200 (173.2)	30000 (1000)	1736.7 (518.3)	20066.7 (832.7)	2866.7 (152.8)	5.6 (0.8)	14.9 (1.0)	27.0 (1.1)	54.7 (2.9)
Chlorsulfuron	+	2833 (2309)	26666.7 (4725.8)	1680 (229.1)	17500 (818.5)	2833.3 (251.7)	5.5 (0.7)	13.3 (0.9)	25.2 (2.6)	52.2 (1.8)
Chlorsulfuron x 2	-	3033.3 (321.5)	24000 (1732.1)	1636.7 (204)	19900 (1967.2)	2866.7 (378.7)	5.4 (0.9)	16.4 (3.9)	34.8 (11.0)	24.5 (1.2)
Chlorsulfuron x 2	+	2900 (173.2)	23333.3 (2081.7)	1453.3 (302.7)	17200 (721.1)	2500 (264.6)	4.6 (0.3)	14.1 (1.7)	28.3 (0.9)	51.0 (3.0)
Triasulfuron	-	3033 (152.7)	26666.7 (577.4)	1880 (310.5)	19600 (458.3)	3033.3 (251.7)	5.8 (0.2)	15.3 (2.1)	26.4 (2.7)	55.3 (1.6)
Triasulfuron	+	256607 (321.5)	26666.7 (4509.3)	1706.7 (376.3)	19566.7 (3092.5)	3066.7 (665.8)	5.0 (0.5)	13.9 (1.1)	25.6 (4.8)	53.3 (3.1)
Triasulfuron x 2	-	3066.7 (208.2)	25000 (2000)	1620 (365.9)	19900 (984.9)	2866.7 (251.7)	5.7 (0.6)	14.6 (0.9)	29.0 (2.1)	56.6 (2.8)
Triasulfuron x 2	+	2933.3 (378.6)	24333.3 (1527.6)	1526.7 (290.2)	17700 (871.8)	2633.3 (450.9)	4.8 (0.3)	13.8 (2.2)	25.9 (6.7)	51.7 (1.8)
Trifluralin	-	3000 (0)	29000 (2645.8)	2066.7 (115.5)	19333.3 (568.6)	3166.7 (57.7)	6.3 (1.2)	15.2 (1.7)	26.8 (2.1)	54.4 (1.9)
Trifluralin	+	2733.3 (115.5)	25666.7 (1527.6)	1486.7 (237.6)	19666.7 (1222.0)	3066.7 (115.5)	5.5 (0.6)	14.5 (3.2)	27.9 (4.6)	52.1 (2.9)
p-values										
Residue		0.426	0.128	0.510	0.914	**0.031**	0.246	0.539	0.254	0.939
Flumetsulam		**0.008**	0.119	**0.001**	**0.003**	**0.041**	**0.007**	**0.026**	0.129	**0.018**
Residue *flumetsulam		0.738	0.588	0.157	0.068	0.319	0.796	0.745	0.725	0.689

4.4 DISCUSSION

The results from this study found that applications of chlorsulfuron and triasulfuron, at either the recommended rate or at double the recommended rate of application, had little effect on the biomass at anthesis, or on the yield of wheat. The results were consistent with those of Lemerle *et al.* (1985) and Brewster and Appleby (1983), who observed no yield loss in wheat after chlorsulfuron was applied at the recommended rate. However, chlorsulfuron applied at 40 g ha^{-1} (three times recommended application rate) reduced the yield of Durati and Songlen wheat cultivars by 15% and 17% respectively when compared to unsprayed controls in New South Wales (Lemerle *et al.,* 1985). Conversely Koscelny *et al.* (1996) found that a tank mix of chlorsulfuron (21 g ha^{-1}) + metsulfuron (210 g ha^{-1}) increased grain yield by 5.7% in Okalahoma, USA and may have been a result of reduced competition or of a direct herbicide effect. However, in the study by Koscelny *et al.* (1996) a reduction in vegetative growth was observed following herbicide application. It was possible that the increased yield observed from chlorsulfuron + metsulfuron application was due to the untreated plants using more resources (moisture and nutrients) earlier in the growing season, so that nutrient availability became a limiting factor when the untreated plants started to fill grain (Koscelny *et al.*, 1996).

Although the herbicides in this experiment did not affect yield, other studies have found that sulfonylurea herbicides may affect nutrient uptake in some cereals. Chlorsulfuron reduced zinc uptake in wheat (McLay and Robson, 1992; Wheal and Rengel, 1997), and uptake of phosphorous and potassium by wheat was shown to decrease following chlorsulfuron and diclofop-methyl application in a glasshouse

experiment (Osborne *et al.,* 1993). Pederson *et al.* (1994), found temporary reductions in phosphorous and zinc concentrations in barley for at least 4 weeks following metsulfuron-methyl application. Temporary minor reductions in manganese, copper, sulfur and potassium were also observed following metsulfuron-methyl application (Pederson *et al.*, 1994). These temporary reductions in plant nutrient concentrations may have resulted from a reduction in fine root hairs and root length (Pederson *et al.*, 1994).

Residues of the sulfonylureas applied in the first year of this experiment reduced the growth of *M. rugosa* in the second year. The residues of chlorsulfuron or triasulfuron reduced medic shoot biomass significantly compared to the first year herbicide treatments where either trifluralin or no herbicide was applied. This result indicated that the sulfonylurea herbicides were still present at levels sufficiently high to adversely affect medic shoot production up to twelve months after their application. Such an effect of sulfonylurea herbicides was consistent with results from other studies.

Gillett and Holloway (1996) found triasulfuron at 1% and 4% of the recommended application rate significantly reduced medic shoot dry matter by 18% and 26% respectively and restricted root penetration. In another study, triasulfuron (4 g a.i. ha^{-1}) or chlorsulfuron (11 g a.i. ha^{-1}) applied to wheat growing on soil with a pH > 8.5, reduced annual medic shoot dry matter by 58% and 78% respectively, and seed production by 54% and 92% respectively, twelve months after application (Evans *et al.*, 1993). The reduction in medic seed yield from triasulfuron or chlorsulfuron reported by Evans *et al.* (1993), was not observed in this study for *M. rugosa* twelve months after application of chlorsulfuron or triasulfuron. This difference may have resulted from the higher soil pH in the study by Evans *et al.* (1993). At higher soil pH values, the

potential exists for enhanced residual herbicide concentrations due to a reduced rate of herbicide breakdown from chemical hydrolysis (Blacklow and Pheloung, 1992; Beyer *et al.*, 1987; Fredrickson and Shea, 1986).

Shoot biomass and seed yield of *M. rugosa* was not affected by flumetsulam in the study discussed in this chapter. The results from this chapter are supported by data from Ewers and Phillips (1993) who found the yields of six pasture legume species were unaffected by flumetsulam. Dickinson *et al.* (1993) found that flumetsulam gave good weed control and high seed yields when applied to annual medic pastures (varieties Parabinga, Harbinger and Parragio) on the Upper Eyre Peninsula, South Australia.

The relative growth rate of *M. rugosa* was reduced by the chlorsulfuron x 2 residue treatment by the final harvest. It is possible that at this stage of growth, the differences between treatments had become large enough to exceed background variation. This stage of the growing season was also the time of greatest biomass production and therefore any inhibition in the growth of plants would become more obvious.

The nutrient analysis conducted showed that the majority of the nutrients were above adequate values for *M truncatula* (Reuter and Robinson, 1997). Manganese and zinc were consistently low across all treatments and would not have contributed to the observed reduction in biomass attributed to herbicide treatments. Different species of medic (i.e. *M truncatula* versus *M. rugosa*), different stages of plant growth were compared (i.e. youngest mature leaf versus whole shoot) and different time of sampling were used in determining the 'adequate levels' of nutrients, and as such the interpretations should be used only as a guideline.

4.5 SUMMARY AND KEY FINDINGS

Residues of chlorsulfuron and triasulfuron reduced shoot biomass production of medic plants in the season subsequent to that in which they were applied. This has important implications for fodder production and grazing management practices when medic pastures are incorporated into rotations with cereals. However, the impact of the sulfonylurea herbicides did not extend to seed yield. The viability of the seed was not determined in this study and it is possible that natural regeneration may be reduced in future years. Further studies are needed to determine the effects, if any, of sulfonylurea herbicides on seed viability. This field trial only investigated the effects on *M. rugosa*, of sulfonylurea residues 12 months after application. If the experiment had continued for another 12 months, the suitability of the recommended plant back period for medic, after chlorsulfuron application on south Australian soils (pH of 7.5 – 8.5), of 24 months (Chambers, 1995) could be determined. The results from this field trial suggest that if this plant back period is not followed, farmers run the risk of a loss in pasture productivity, accompanied by a loss in feed for pasture animals, and a potential loss of nutrient availability for future crops (Peoples *et al.*, 1992) due to reduced medic residues for incorporation into the soil. On average, pastures in southern Australia fix 20 – 25 kg N tonne^{-1} of shoot biomass (Peoples and Baldock, 2001). Therefore the reduction in shoot biomass from herbicide application will decrease the amount of biologically fixed nitrogen incorporated into the medic/cereal rotation.

Flumetsulam did not effect either biomass or yield of medic and was found to be safe for use at the recommended application rate on medic.

CHAPTER 5

EFFECTS OF ALS-INHIBITING HERBICIDES ON CHICKPEA GROWTH AND PRODUCTION IN THE FIELD

5.1 INTRODUCTION

The inclusion of grain legumes in cropping rotations helps maintain soil fertility and break cereal disease cycles (Reeves, 1987). Grain legumes are also high protein cash crops that allow for a greater diversification in crop rotations (Tow and Schultz, 1991). Mineral nitrogen levels in the year following production of a grain legume are usually higher than after a cereal crop, although this depends on factors such as the amount of nitrogen removed in harvested seed, fate of crop residues, rate of mineralisation of organic nitrogen, and extent of nitrogen losses by leaching and denitrification (Tow and Schultz, 1991).

Grain legume production in Australia has increased over the past 25 years to approximately 2 x 10^6 tonnes per year (Siddique and Sykes, 1997). Production of chickpea increased rapidly from the time of its introduction to Australia in the 1890's to the 1990's when production in Queensland and New South Wales was restricted by drought (Siddique and Sykes, 1997). Future expansion in Australian grain legume production is likely to be dominated by chickpea and faba bean industries (Siddique and Sykes, 1997). In 1996/97, 12,000 hectares were sown with chickpeas and 17,000 tonnes of chickpeas were harvested in South Australia (PIRSA, 1998). A further 210,000 ha were sown to chickpea in Victoria (90,000 ha), Western Australia (60,000 ha), New South Wales (30,000 ha) and Queensland (30,000 ha) (Siddique and Sykes,

1997). The amount of nitrogen fixed by chickpeas varies throughout Australia with measured values ranging between 15 and 124 kg N ha^{-1} (Armstrong *et al.*, 1997; Horn *et al.*, 1996b; Evans *et al.*, 1989). These differences may be due to soil type, rainfall, disease, phosphorous uptake and adequate rhizobial inoculation.

The choice of grain legume crop in South Australia is largely determined by rainfall and soil type (Tow and Schultz, 1991). Chickpeas are best suited to clay or loam soils with a pH range of 6.0-9.0 and an annual rainfall of 350-400 mm (Lamb and Podder, 1992).

Herbicides are used in grain legume crops to control grass weeds, which reduces cereal root pathogens and grass weeds in the following cereal crop (Tow and Schultz, 1991). Broad leaf weeds can reduce chickpea yield and need to be controlled (Lamb and Podder, 1992). The ALS-inhibiting herbicides, flumetsulam (Broadstrike ®) and imazethapyr (Spinnaker ®), can be used in chickpea crops to control broadleaf weeds such as Bedstraw (*Galium tricornutum*), Mustard (*Sisymbrium orientale*), and Wireweed (*Polygonum aviculare*) (PISA, 1996). A yellowing of leaves and stunting of growth has been observed in some grain legumes, including chickpeas, following the use of these herbicides (DowElanco herbicide label). However, chickpeas were found to be tolerant, in terms of seedling growth and grain yield, to both flumetsulam and imazethapyr at pH 5.5 – 7.8 in southern Queensland (Barnes *et al.*, 1996).

Sulfonylureas such as chlorsulfuron (Glean®), triasulfuron (Logran®) and metsulfuron methyl (Ally®) are widely used in cereal crops to control broadleaf weeds and some grasses. Yellowing of shoot tips, inhibition of root growth, yield reduction and premature death were observed in chickpea plants twelve months after

chlorsulfuron application on alkaline soils in New South Wales, Australia (Ferris *et al.*, 1992). A reduction in chickpea shoot matter and grain yield (15%) was observed 8 – 14 months after chlorsulfuron application at pH 7.4 in Greece (Efthimiadis *et al.*, 1989).

The experiment in Chapter 3 found that shoot and root biomass and relative growth rates of chickpeas grown in a glasshouse were reduced by flumetsulam. Glasshouse or pot trials are often unrepresentative of field conditions. In herbicide studies, roots of plants grown in pots will be in contact with higher concentrations of the herbicide than those grown in the field. The field trial in this chapter will determine if reductions in biomass and relative growth rates are also observed in the field. The pot trial from Chapter 3 will be extended to include the effects of imazethapyr and residues of chlorsulfuron in addition to flumetsulam, on chickpeas.

The objectives of this experiment were to determine the effects on shoot growth, seed yield and nitrogen fixation of chickpeas from the following:

1. Chlorsulfuron residues;
2. In-crop application of flumetsulam or imazethapyr;
3. Cumulative effects of chlorsulfuron residues and in-crop applications of flumetsulam or imazethapyr.

5.2 MATERIALS AND METHODS

5.2.1 Site selection and soil characteristics

The field trial was established on a Sodic, Supracalcic, Red Chromosol soil (Soil Taxonomy (USDA 1994): Natrixeralf) located at the Roseworthy Campus of the

University of Adelaide. The site was selected based on alkaline soil, as sulfonylureas persist under these conditions, with no sulfonylurea use in the past seven years. Table 5.1 gives details of soil characteristics.

Table 5.1: Soil characteristics for paddock West 10 at the Roseworthy campus of the University of Adelaide.

Soil characteristic	Value
pH (water)	8.3
pH (calcium chloride)	7.7
Organic carbon	1.21%
Extractable phosphorous	20 mg kg^{-1}
Extractable potassium	406 mg kg^{-1}
Extractable zinc	1.8 mg kg^{-1}
Soil salinity	
EC (1:5)	0.15 ds m^{-1}
Ece (est)	1.5 ds m^{-1}
Free lime	Moderate
Soil texture	clay loam
Cation exchange capacity	28.03 mequiv $100g^{-1}$

5.2.2 Treatments

Four rates of chlorsulfuron were applied to the soil prior to sowing chickpeas. The rates were selected to represent residual levels that may remain twelve months after chlorsulfuron application (Table 5.2). Chlorsulfuron was sprayed on 30th May 1997 using a 10m boom. The trial was cultivated (20 cm) to incorporate chlorsulfuron into the soil in order to as closely as possible resemble residual conditions.

Table 5.2: Application rates of chlorsulfuron for field trial investigating the effects of ALS-inhibiting herbicides on chickpea.

	g ha^{-1}	g ai ha^{-1}	% of rec. rate
Nil	0	0	0
Low	1	0.75	5
Medium	2	1.5	10
High	4	3	20

Desavic chickpea seeds were inoculated with the *Rhizobium* Nitrogerm 100 Group N chickpea inoculant manufactured by Biocare Technology Pty. Ltd on the 4th of June 1997 and were sown on the 5th June at 100kg ha^{-1}. Super phosphate with zinc was applied at 100kg ha^{-1} at the time of sowing. The row spacing was 18 cm and a 14-row drill was used. Oats were hand sown on the 9th of June along the borders of each plot as reference plants for use in ^{15}N natural abundance nitrogen fixation estimates.

Imazethapyr (2-[4,5-dihydro-4-methyl-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid) was applied at a rate of 120 ml ha^{-1} (29 g ai ha^{-1}) on the 10th June 1997. Flumetsulam was applied at 25 g ha^{-1} (20 g ai ha^{-1}) on the 23rd July 1997, when the chickpeas had reached the four to six branch stage. Both herbicides were applied at the recommended application rates. Grass weeds were sprayed in August with Targa (94 g L^{-1} quizalofop-p-ethyl) at 350 ml ha^{-1}. The hand sown oats (reference plants) were covered with plastic pots to avoid damage. A 'control' of no additional herbicide was also included.

5.2.3 Experimental design

The experiment was set up as a strip plot design. Four rates of chlorsulfuron, representing residual levels, (Table 5.2) were first applied in a randomised block design (Figure 5.1). Then flumetsulam, imazethapyr or no additional herbicide treatments were applied over the chlorsulfuron treatments after sowing the chickpeas (Figure 5.1). Each combination of herbicide treatments was replicated once in each of four blocks (Figure 5.1). Each chlorsulfuron by additional herbicide plot measured 10m x 15m (Figure 5.1).

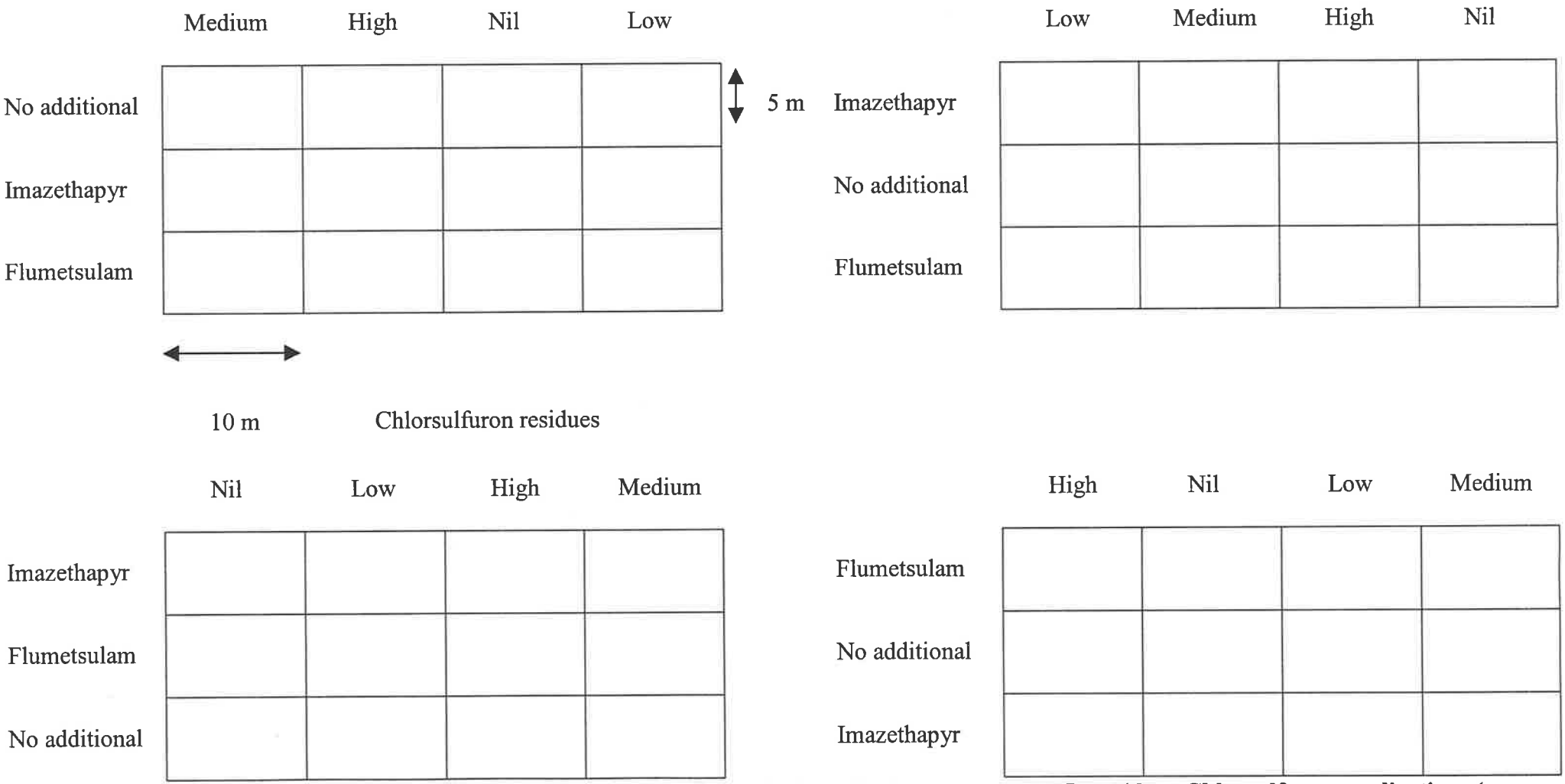


Figure 5.1: Field trial plan showing herbicide application (Strip plot design). Each plot measures 5m x 10m. Chlorsulfuron applications (see Table 5.2 for application rates) were applied down each replicate. The herbicides (flumetsulam and imazethapyr) were applied across each replicate.

5.2.4 Sampling and measurements

Samples were taken for shoot biomass throughout the year and seed yield at the end of the growing season (Table 5.3). After collection, shoots were dried in an air-draught oven at 60°C for 48 hours. Following drying, the shoots were weighed for shoot biomass. This data was used to calculate absolute (AGR) and relative (RGR) growth rates using equations 5.1 and 5.2 respectively.

$$AGR = \frac{\text{biomass at time 2 - biomass at time 1}}{\text{time 2 - time 1}} \quad (5.1)$$

$$RGR = \frac{\ln \text{biomass at time 2 - biomass at time 1}}{\text{time 2 - time 1}} \quad (5.2)$$

Leaf area index was measured at flowering, using a LI-COR LAI 2000 plant canopy analyser. The LAI – 2000 uses measurements taken above and below the canopy to determine canopy light interception at five angles, from which LAI is computed using a model of radiative transfer (LI-COR LAI – 2000 operation manual).

Chickpea shoot samples collected at flowering (peak biomass) were analysed for total nitrogen and ^{15}N natural abundance analyses and macro and micro nutrient concentrations. Nutrient concentrations were measured using ICP analysis. ICP analyses were completed by digesting ground (<2mm) plant material in nitric acid and determining the concentration in the digests using Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) (Zarcinas and Cartwright, 1987). Nutrient concentrations were determined to ensure that nutrient concentrations in the shoots were within adequate guidelines for chickpea plants and to determine whether the herbicides in question affected nutrient concentrations.

Chickpea plant samples for total carbon/nitrogen analyses were ground using a Makla Mill (< 2 mm) and were analysed using a LECO nitrogen analyser (LECO CN-2000).

The ^{15}N natural abundance method has become the most common means of assessing nitrogen fixation in Australia (Unkovich *et al.*, 1997; Unkovich *et al.*, 1994; Peoples *et al.*, 1991). ^{15}N is a stable isotope of nitrogen and occurs in the atmosphere at a constant abundance of 0.3663 atoms % (Peoples *et al.*, 1989). If the ^{15}N abundance in plant-available soil nitrogen is higher than this, an estimate of the proportion of legume nitrogen derived from each source can be made (Peoples *et al.*, 1989). The ^{15}N abundance of plant available nitrogen residing in the soil is obtained by analysing a non nitrogen fixing reference plant (Peoples and Herridge, 1990; Peoples *et al.*, 1989). In this field trial, oats sown around the border of each plot were used as the reference plants. Chickpea and oat shoot samples were dried at 60°C and ground (< 2mm) initially using a Makla Mill, subsampled and then finely ground using a Lab Technics Laboratory Pulverising Mill. Finely ground shoot material was weighed and sealed in tin capsules, then combusted and the reaction products were separated by Gas Chromatograph to give a pulse of pure nitrogen. The total amount of N and the amount of ^{15}N were quantified by isotope mass spectrometry (20-20. Europa Scientific Crewe, UK). ^{15}N natural abundances are normally expressed in a relative notation , δ , which converts the decimal atom % to a more manageable integer and was calculated by equation 5.3 (Unkovich *et al.*, 1994):

$$\delta^{15}N\ (^{0}/_{00}) = \frac{\text{atom\% } ^{15}N \text{ sample} - \text{atom\% } ^{15}N \text{ air}}{\text{atom\% } ^{15}N \text{ air}} \times 1000 \qquad (5.3)$$

The percentage of plant nitrogen derived from the atmosphere (%Ndfa) was calculated according to the equation 5.4:

$$\%Ndfa = \frac{\delta^{15}N \text{ reference plant} - \delta^{15}N \text{ legume}}{\delta^{15}N \text{ reference plant} - B} \times 100 \qquad (5.4)$$

Where B refers to the $\delta^{15}N$ of the nodulated legume grown in the presence of rhizobia and with no source of plant - available inorganic nitrogen (Unkovich *et al.*, 1997). In this experiment, an appropriate B value was obtained by measuring the $\delta^{15}N$ of chickpea shoots grown in a glasshouse until flowering in vermiculite containing no nitrogen.

Rainfall data was collected using a tipping bucket attached to an automated weather station, from a paddock adjacent to the one used for the field trial.

Table 5.3: Sampling times and methods of collection for field trial.

Date	Time	Method	Samples taken
22/07/97	Pre – flumetsulam application	3 quadrats (40x40cm) bulked	Shoots
25/08/97	Post – flumetsulam (not complete sampling due to excessive grass weeds)	10 random plant samples – collected mainly for photographs	Whole plants
18/09/97	Post- flumetsulam (after removing grass weeds)	Plants per metre row	Shoots
9/10/97	Flowering	2 quadrats (1m x 25cm) taken across rows	Shoots, Leaf area index,
7/01/98	Hand harvest	1 quadrat (1m x 25cm) taken across rows	Pods
12/01/98	Machine harvest	Kew plot harvester	Seed yield

5.2.5 Data analysis and interpretation

Significant (@ $\alpha = 0.05$) results were identified by analysis of variance, that included main effects of chlorsulfuron, additional herbicides, harvest time, and their

interactions, using Genstat 5 Release 4.1 (Payne, 1993). Chlorsulfuron effects refer to residual effects of applying 0, 5, 10 and 20% of the recommended application rate of chlorsulfuron prior to sowing the chickpeas. Additional herbicide effects refer to the effects of the additional herbicides applied to the chickpeas: imazethapyr, flumetsulam, or no additional herbicide. Where the analysis of variance identified significant treatment effects, Tukey's H.S.D. test was performed to identify which components of each treatment differed significantly. Graphs of significant treatment effects and interactions show means obtained from an average of all other treatments.

Data for ^{15}N analysis was unbalanced because one replicate for one combination of treatments was missing and the analysis of variance could not be used. In this case Restricted Maximum Likelihood (REML) which can handle unbalanced data was used. The purpose of the REML was to determine the treatment effects when there were several sources of variability. Using Wald's test, which is approximately Chi-squared distributed, conclusions can be made about the importance of treatment effects. Results are significant when the Wald's statistic is greater than the critical value for Chi-square distribution. When significant results were found, Tukey's H.S.D. test was used to determine significant differences. Only significant effects will be reported in the text.

5.3 RESULTS

5.3.1 Shoot Biomass

There were significant two way interactions between sampling time and chlorsulfuron (Table 5.4, Figure 5.2) and sampling time and herbicide applications on shoot biomass (Table 5.4, Figure 5.3). In both cases, shoot biomass increased over the growing season. Chickpea shoot biomass was reduced by increasing levels of

chlorsulfuron and this reduction increased over time (Figure 5.2). At flowering, 20%, 10% and 5% chlorsulfuron treatments had reduced shoot biomass by 49.64%, 36.3 % and 22.24% respectively compared to the nil chlorsulfuron treatment (Figure 5.2).

The no additional herbicide, flumetsulam and imazethapyr treatments differed in their affects on chickpea shoot biomass over time. Shoot biomass of the no additional herbicide and flumetsulam treatments increased over the growing season, while the imazethapyr treatment increased in biomass by only 12% between 81 and 125 days (Figure 5.3). At flowering, imazethapyr and flumetsulam application had reduced shoot biomass by 52.51% and 24.38% respectively when compared to the plots with no additional herbicide (Figure 5.3).

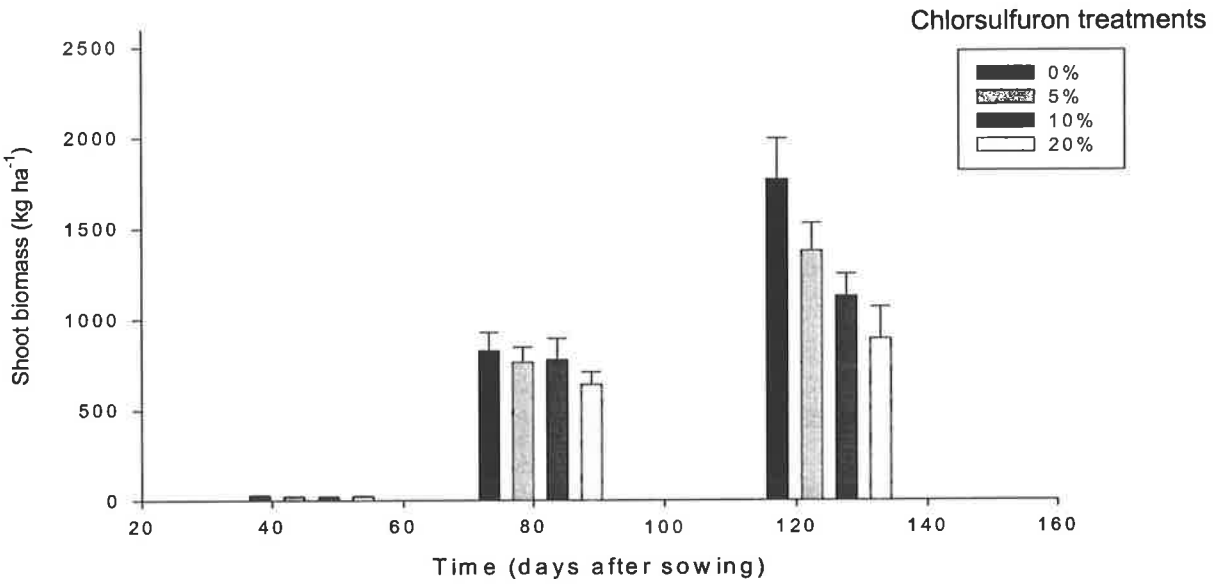


Figure 5.2: The effects of chlorsulfuron (applied at 0, 5, 10 and 20% of the recommended application rate) and harvest time on shoot biomass of chickpeas. Bars indicate standard error of mean.

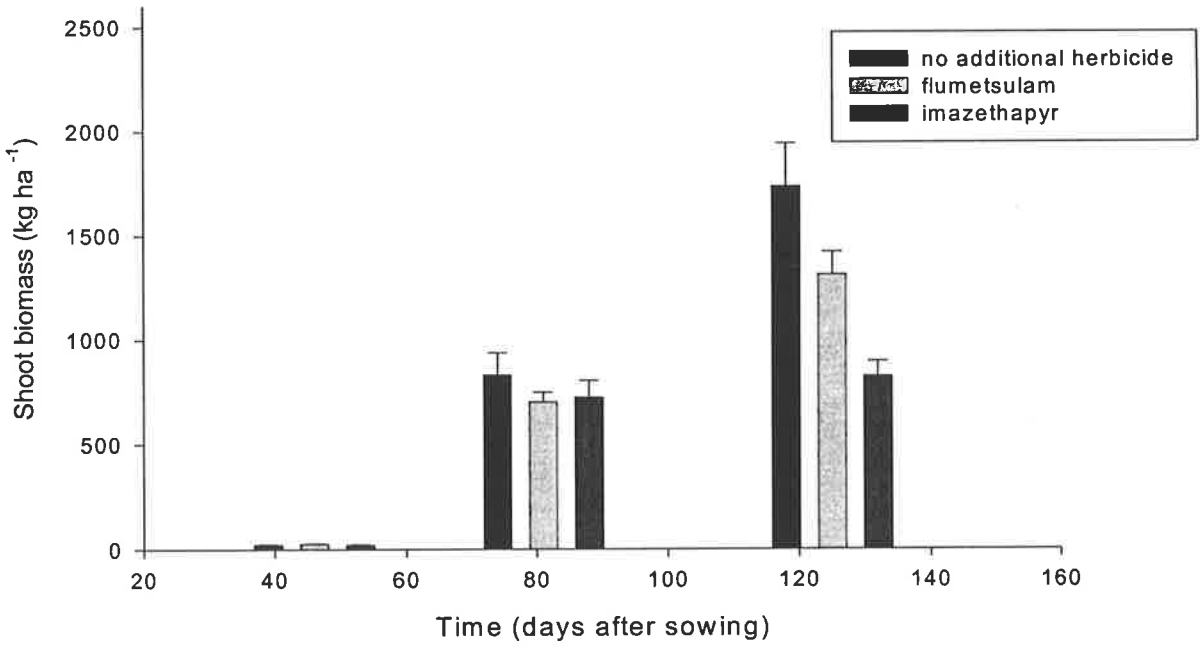


Figure 5.3. The effects of flumetsulam (20 g ai ha^{-1}), imazethapyr (29 g ai ha^{-1}) applied to soils with residual levels of chlorsulfuron, and sampling time on chickpeas shoot biomass. Bars indicate standard error of mean.

Table 5.4: Results of analysis of variance for effects of chlorsulfuron, flumetsulam and imazethapyr on chickpea shoot biomass, leaf area index and yield. Significant (α=0.05) values are in bold. * = interaction.

variable	Source	P value
Shoot weight	Chlorsulfuron	**<0.001**
	Herbicide	0.090
	Time	**<0.001**
	Chlorsulfuron*herbicide	0.389
	Chlorsulfuron*time	**<0.001**
	Herbicide*time	**0.010**
	Chlorsulfuron*herbicide*time	0.686
Leaf area index	Chlorsulfuron	**0.005**
	Herbicide	**<0.001**
	Herbicide*chlorsulfuron	0.098
Yield (kg ha^{-1})	Chlorsulfuron	**0.011**
	Herbicide	0.078
	Chlorsulfuron*herbicide	**0.004**

5.3.2 Leaf Area Index

There were significant main effects of chlorsulfuron (Table 5.4, Figure 5.4) and additional herbicide (Table 5.4, Figure 5.5). The nil and 5% rates of chlorsulfuron treatments had similar LAI's. The 10% and 20% rates of chlorsulfuron reduced the LAI of chickpea shoots by 37% and 46% respectively compared to the nil chlorsulfuron treatment (Figure 5.4).

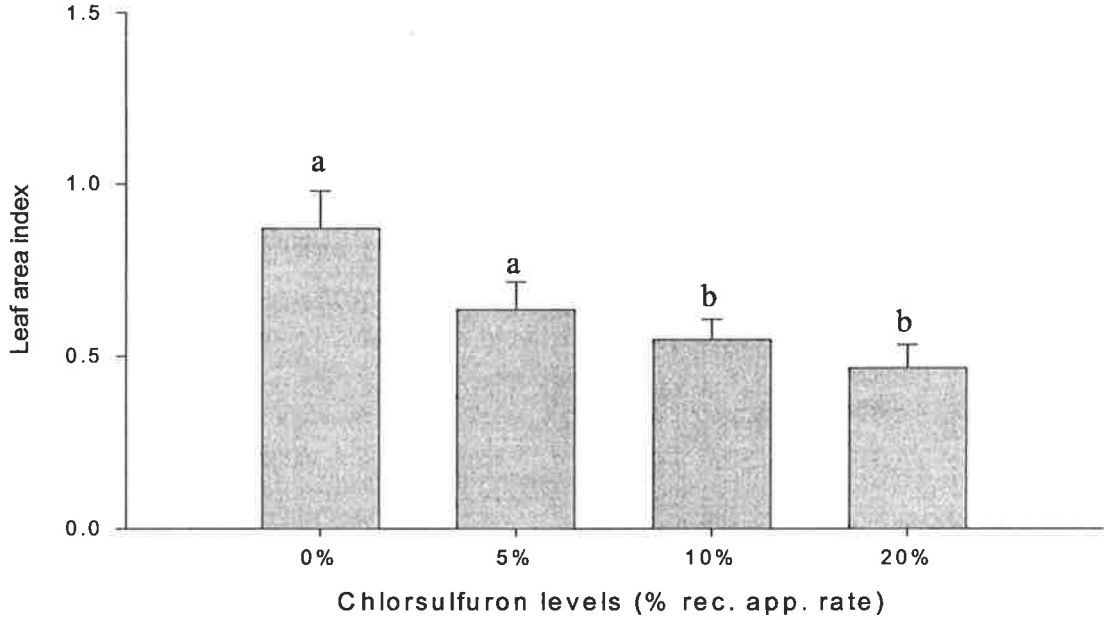


Figure 5.4. Significant effects of chlorsulfuron applied at 0, 5, 10 and 20% or recommended application rate on leaf area index of chickpeas at flowering. Bars indicate standard error of mean. Similar letters indicate similar values as determined by Tukey's H.S.D test.

The no additional herbicide treatment had the highest leaf area index and was significantly different from both flumetsulam and imazethapyr (Figure 5.5). Imazethapyr and flumetsulam reduced LAI of chickpea shoots by 25 and 42% respectively compared to the no additional herbicide treatment (Figure 5.5).

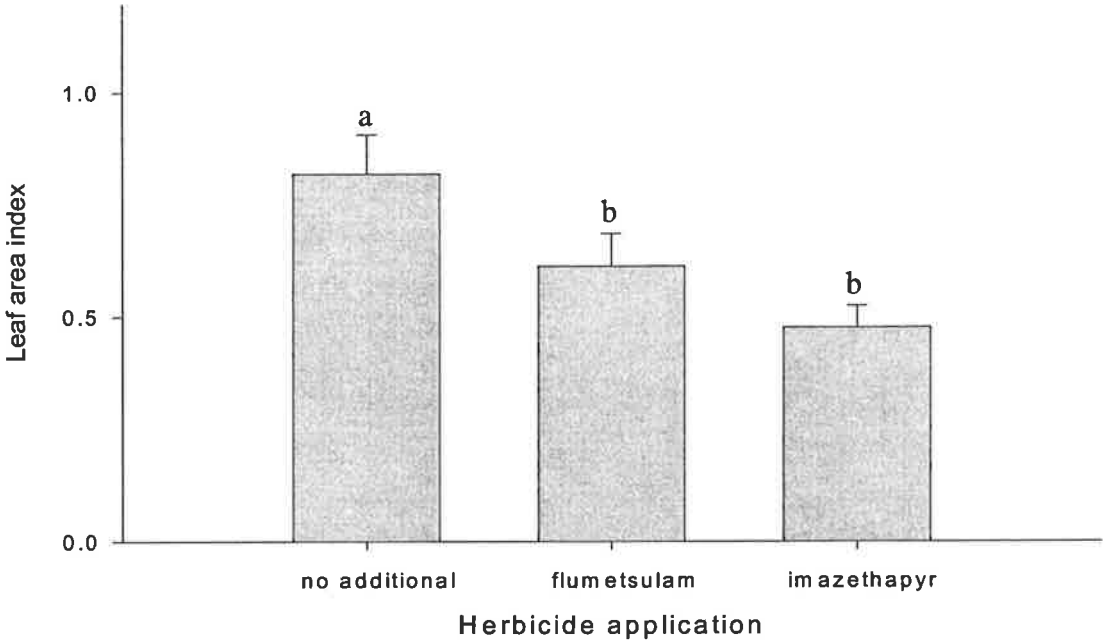


Figure 5.5. The effects of flumetsulam (20 g ai ha^{-1}) and imazethapyr (29 g ai ha^{-1}) on leaf area index of chickpeas at flowering. Bars indicate standard error of mean. Similar letters indicate similar values as determined by Tukey's H.S.D test.

5.3.3 Grain yield

Grain yield was affected by a significant interaction between residual levels of chlorsulfuron and applications of flumetsulam and imazethapyr (Table 5.4; Figure 5.6). Chickpea yield from flumetsulam and imazethapyr treatments increased from nil chlorsulfuron to the 5% rate and then declined in the presence of higher rates of chlorsulfuron (Figure 5.6). In the no additional herbicide treatments, yield increased up to the 10% level of chlorsulfuron and declined with the 20% application (Figure 5.6.).

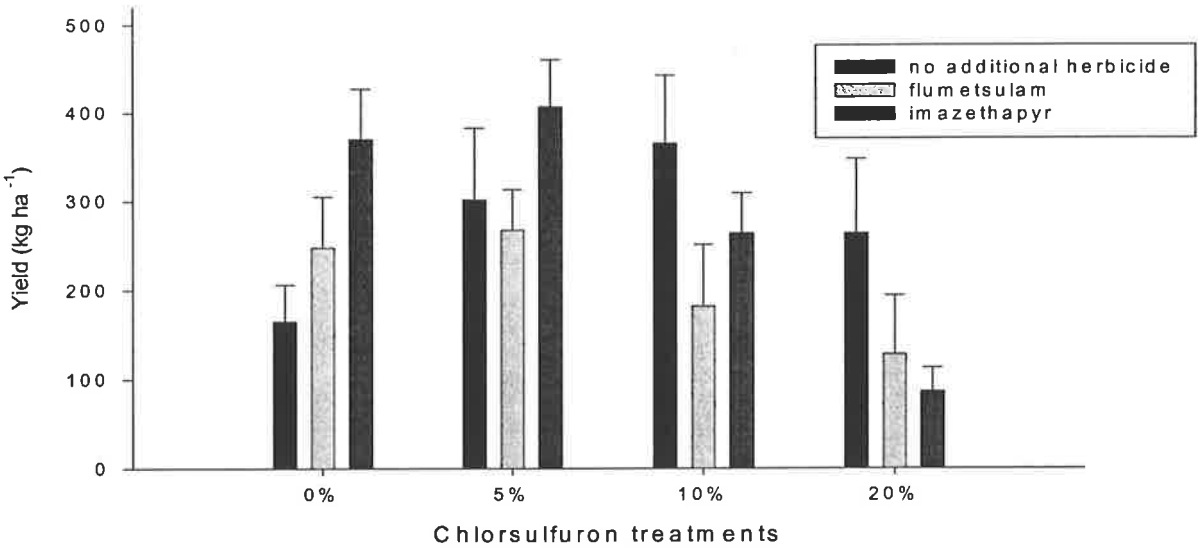


Figure 5.6: The effects of chlorsulfuron applied at 0, 5, 10 and 20% of the recommended application rate and additional applications (at the recommended rate) of flumetsulam and imazethapyr on chickpea yield. Bars indicate standard error of mean.

5.3.4 Absolute and relative growth rates

Chlorsulfuron and the in-crop herbicide treatments interacted to affect the absolute growth rate of chickpea plants between sowing and the first sampling at 27 days (Table 5.5, Figure 5.7). Chickpeas in the imazethapyr treatment had a consistent absolute growth rate across all rates of chlorsulfuron, while chickpeas treated with flumetsulam or no additional herbicide varied. In the absence of additional herbicide, the highest growth rate in the control plots was in the 5% chlorsulfuron treatment, with the lowest in the 10% chlorsulfuron (Figure 4.6). Plants treated with flumetsulam had the highest growth rates (between the first and second sampling) in the nil chlorsulfuron treatment and the lowest in the 5% treatment.

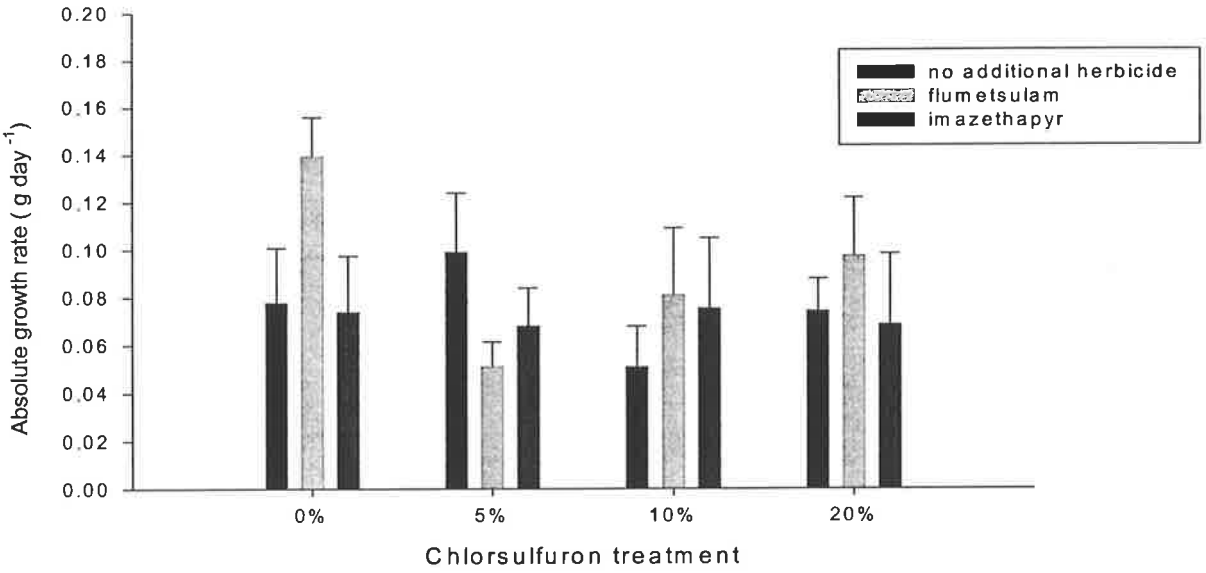


Figure 5.7: The effects of chlorsulfuron (0, 5, 10 and 20% of rec. app. rate) and flumetsulam and imazethapyr at recommended application rate on absolute growth rate of chickpeas between 0 and 27 days after sowing. Bars indicate standard error of mean.

Chlorsulfuron reduced the absolute growth rates of chickpeas between both the first (27 days after sowing) and second (85 days after sowing) samplings (Figure 5.8), and the second (85 days after sowing) and third (106 days after sowing) samplings (Figure 5.9). Between 27 and 85 days after sowing nil, 5% and 10% chlorsulfuron had similar absolute growth rates. The application of 20% chlorsulfuron significantly reduced the absolute growth rates of chickpeas by 23% when compared to the nil chlorsulfuron treatment (Figure 5.8).

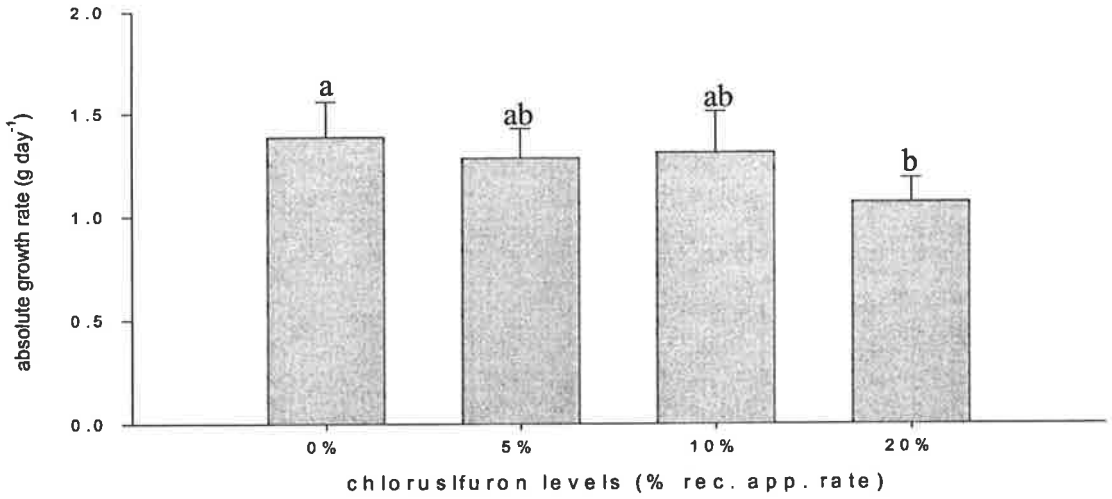


Figure 5.8: Chlorsulfuron (0, 5, 10 and 20% rec. app. rate) effects on the absolute growth rate of chickpeas between 27 and 85 days after sowing. Bars indicate standard error of mean. Similar letters represent similar values as determined by Tukey's H.S.D. test.

Between 85 and 106 days, only the nil chlorsulfuron treatment had a positive absolute growth rate. All chlorsulfuron treatments had a negative absolute growth rate, indicating senescence. The absolute growth rates of the 10 and 20% rates of chlorsulfuron were 480% and 450% respectively lower than the nil plots (Figure 5.9).

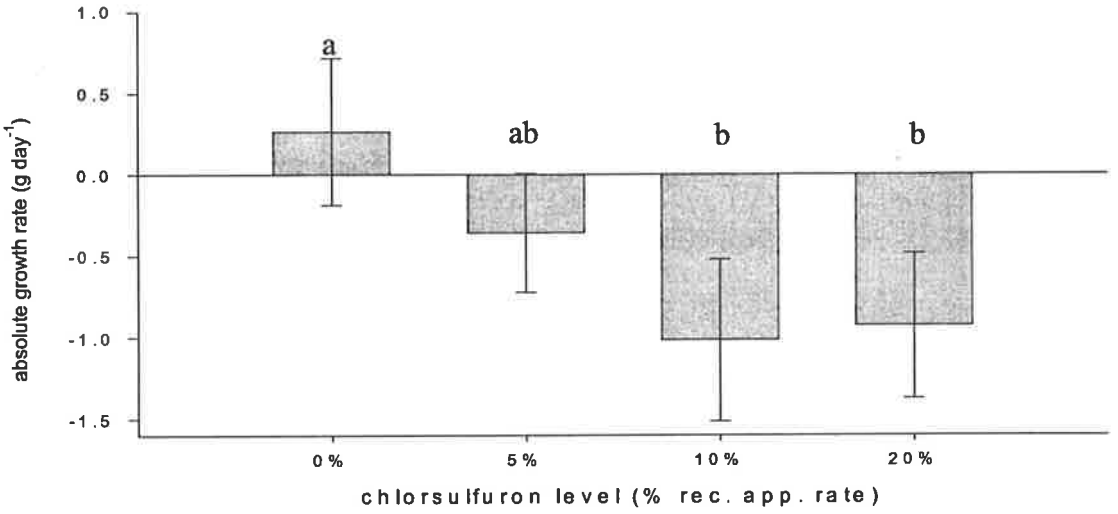


Figure 5.9: Chlorsulfuron (0, 5, 10 and 20% or the recommended application rate) effects on absolute growth rates of chickpeas between 85 and 106 days after sowing. Bars indicate standard error of mean. Similar letters indicate similar values as determined by Tukey's H.S.D test.

The relative growth rates of chickpea plants treated with all rates of chlorsulfuron were negative (indicating senescence) between 85 and 106 days after sowing (Figure 5.10). The relative growth rate of the nil chlorsulfuron treatment differed significantly from 10 and 20% chlorsulfuron, but was not significantly different from the 5% rate (Figure 5.10).

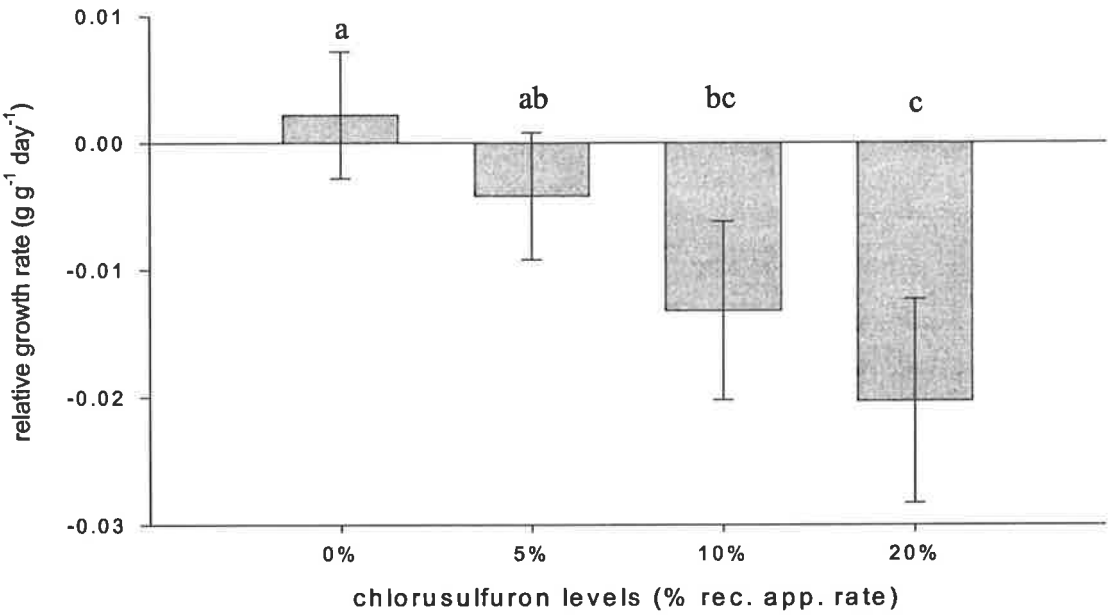


Figure 5.10: The effects of chlorsulfuron (0,5,10 and 20% of the recommended application rate) on relative growth rates of chickpea plants between the 85 and 106 days after sowing. Bars indicate standard error of mean. Similar letters indicate similar values as determined by Tukey's H.S.D. test.

Table 5.5: Analysis of Variance results for effects of ALS-inhibiting herbicides on absolute and relative growth rates (0 – 27), (27 – 85) and (85 – 106) days after sowing.

Variable	Source	p-value
Absolute growth rate (0-27)	Chlorsulfuron	**0.014**
	Herbicide	0.688
	Chlorsulfuron*Herbicide	**0.042**
Absolute growth rate (27-85)	Chlorsulfuron	**0.017**
	Herbicide	0.695
	Chlorsulfuron*Herbicide	0.637
Absolute growth rate (85-106)	Chlorsulfuron	**0.023**
	Herbicide	**0.072**
	Chlorsulfuron*Herbicide	0.608
Relative growth rate (27-85)	Chlorsulfuron	0.749
	Herbicide	0.621
	Chlorsulfuron*Herbicide	0.428
Relative growth rate (85-106)	Chlorsulfuron	**<0.001**
	Herbicide	0.063
	Chlorsulfuron*Herbicide	0.495

5.3.5 Plant Nitrogen or Total Nitrogen

Total nitrogen (%N) of chickpea shoots at flowering was not affected by an interaction between chlorsulfuron and herbicide (p=0.769) or main effects of chlorsulfuron (p=0.171) or herbicides (p=0.661). Total shoot nitrogen (biomass * %N/100) found in chickpeas and the amount of biologically fixed nitrogen of these plants at flowering are summarised in Table 5.6. Biologically fixed nitrogen per hectare was calculated using the % Ndfa and the total shoot nitrogen per hectare found in chickpea shoots. Using shoot biomass the amount of biologically fixed nitrogen per

tonne of shoot biomass was calculated (Table 5.6). The results from the restricted maximum likelihood (REML) statistical analysis are presented in Table 5.7.

Table 5.6: Plant nitrogen content and biologically fixed nitrogen in chickpea shoots at flowering.

Herbicide	Chlorsulfuron	Total N (%)	Total shoot N (Kg N ha^{-1})	Ndfa (%)	Biologically fixed N	
					(Kg N_{fix} ha^{-1})	(Kg N_{fix} tonne shoot $matter^{-1}$)
Control	0%	2.53	62	51.64	31	13
	5%	2.72	48	54.23	24	14
	10%	2.50	37	50.86	19	13
	20%	2.41	34	44.95	14	11
Flumetsulam	0%	2.59	44	53.18	23	14
	5%	2.60	38	51.18	20	14
	10%	2.54	32	20.38	16	13
	20%	2.10	17	38.43	7	8
Imazethapyr	0%	2.95	35	20.82	9	6
	5%	2.56	25	31.24	8	8
	10%	2.6	18	29.96	6	9
	20%	2.26	12	31.69	4	7

There were significant main effects of both chlorsulfuron and additional herbicides for total nitrogen in chickpea shoots per hectare (Table 5.7, Figure 5.11 and Figure 5.12). The 10% and 20% rates of chlorsulfuron resulted in a significant reduction of 38% and 56% respectively in total shoot nitrogen of chickpeas compared to the nil chlorsulfuron treatment (Figure 5.11). The nil and 5% rates were not significantly different as determined by Tukey's H.S.D test (Figure 5.11).

Table 5.7: REML results for plant nitrogen and biologically fixed nitrogen data showing Wald statistic and critical value for chi-square distribution at 5% level.

Variable	Treatment	Wald Statistic	Crit. value for chi-square distribution	Significance
Total shoot N (Kg N ha^{-1})	Chlorsulfuron	21.9	7.81	**Significant**
	Herbicide	20.1	5.99	**Significant**
	Chlorsulfuron *Herbicide	1.3	12.59	Not significant
Biologically fixed N (Kg N fixed ha^{-1})	Chlorsulfuron	45.7	7.81	**Significant**
	Herbicide	73.0	5.99	**Significant**
	Chlorsulfuron *Herbicide	10.2	12.59	Not significant
Biologically fixed N(kg N fixed tonne shoot matter)	Chlorsulfuron	4.7	7.81	Not significant
	Herbicide	18.6	5.99	**Significant**
	Chlorsulfuron *Herbicide	4.4	12.59	Not significant

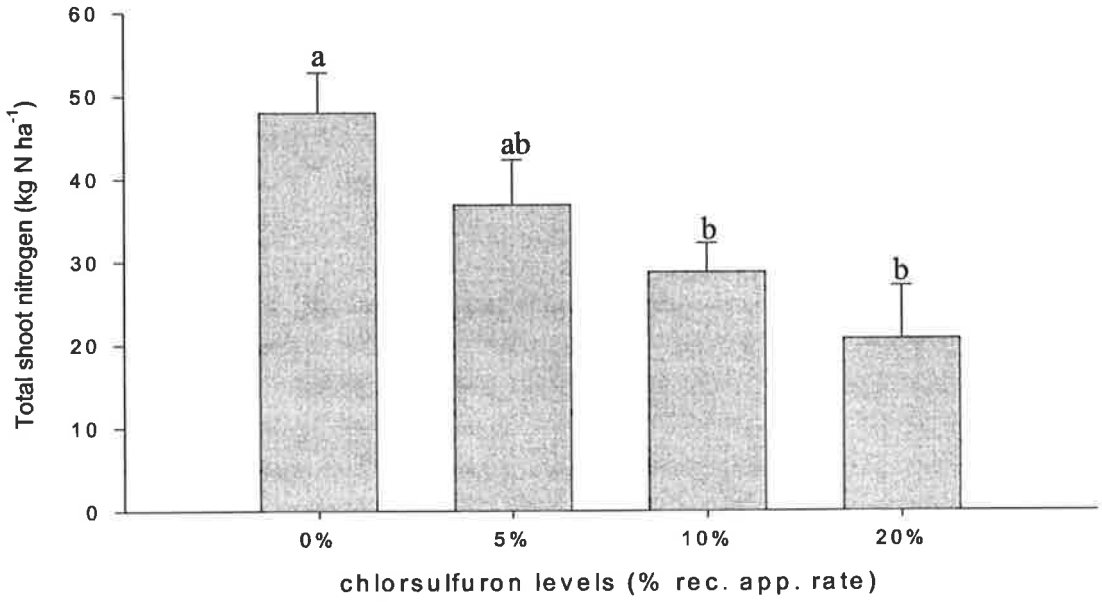


Figure 5.11: Effects of chlorsulfuron (0, 5, 10 and 20% of the recommended application rate) on the amount of nitrogen in chickpea shoots at flowering. Bars indicate standard error of mean. Similar letters indicate results that do not differ significantly as determined by Tukey's H.S.D. test.

Imazethapyr reduced the total shoot nitrogen at flowering by 52% compared to the no additional herbicide treatment (Figure 5.12). Flumetsulam was not significantly different to either the no additional herbicide or imazethapyr treatments (Figure 5.12).

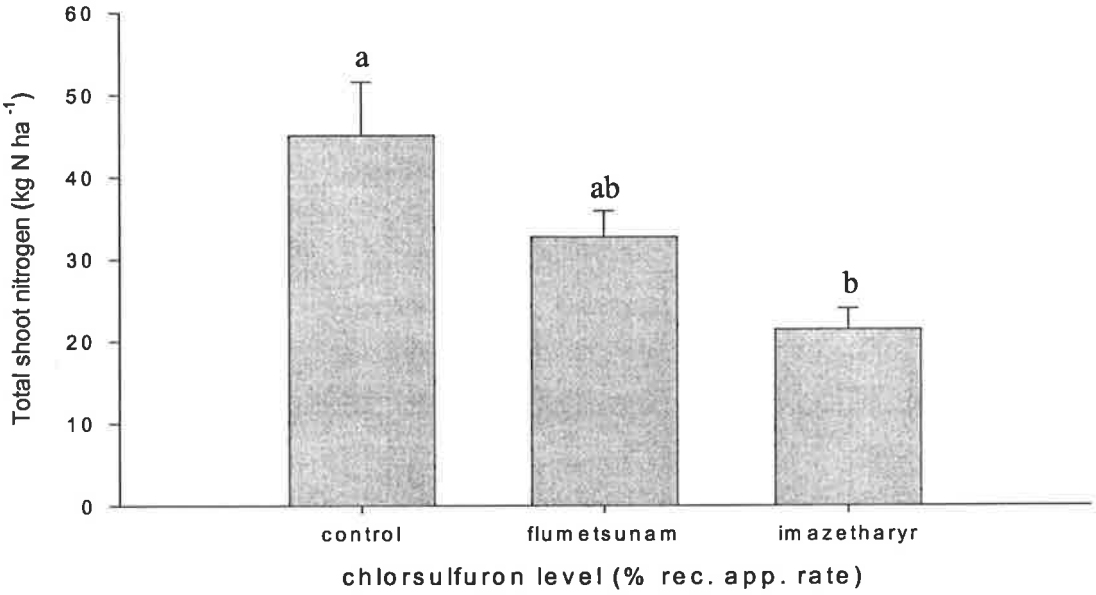


Figure 5.12: Herbicide (flumetsulam and imazethapyr) effects on total shoot nitrogen of chickpea at flowering. Bars indicate standard error of mean. Similar letters indicate values that are not significantly different as determined by Tukey's H.S.D. test.

There were significant main effects of chlorsulfuron (Table 5.7; Figure 5.13) and additional herbicides (Table 5.7; Figure 5.14) on biologically fixed nitrogen per hectare. The 10 and 20% application rates of chlorsulfuron significantly reduced biologically fixed nitrogen by 34 and 60% respectively compared to the nil chlorsulfuron rate (Figure 5.13). The amount of biologically fixed nitrogen of chickpeas grown in the 5% rate of chlorsulfuron was similar to both nil and 10%, with 10% similar to 20% (Figure 5.13).

Imazethapyr and flumetsulam significantly (Table 5.7) reduced the amount of biologically fixed nitrogen by 71% and 24% respectively when compared to those with no additional herbicide (Figure 5.14).

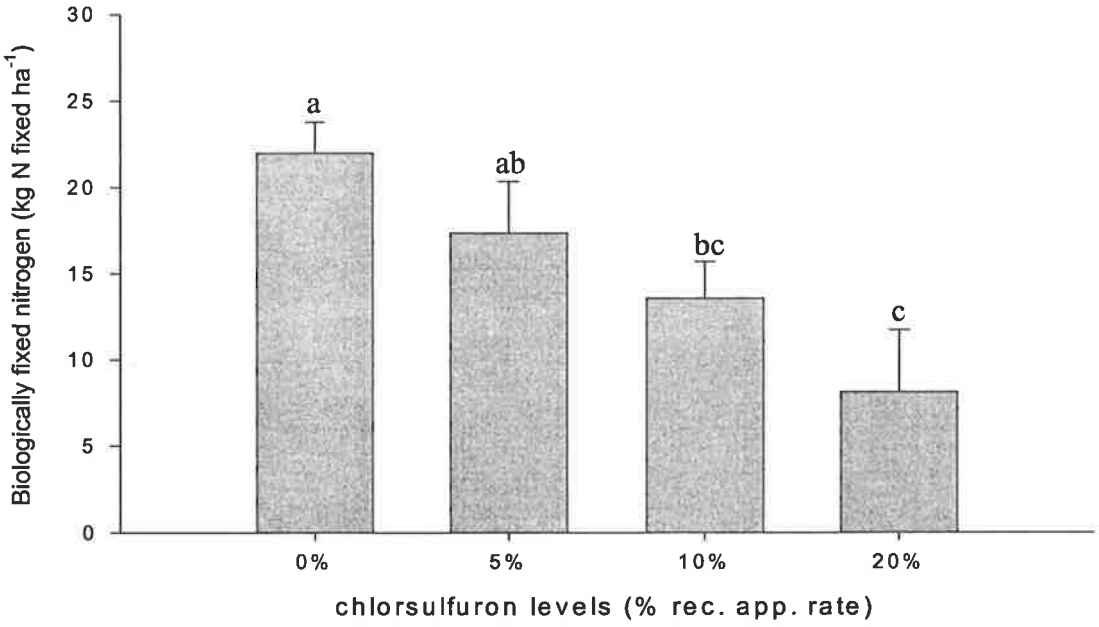


Figure 5.13: The effects of chlorsulfuron (0, 5, 10 and 20% of the recommended application rate) on biologically fixed nitrogen by chickpea plants. Bars indicate standard error of mean. Similar letters indicate statistical similarity as determined by Tukey's H.S.D test.

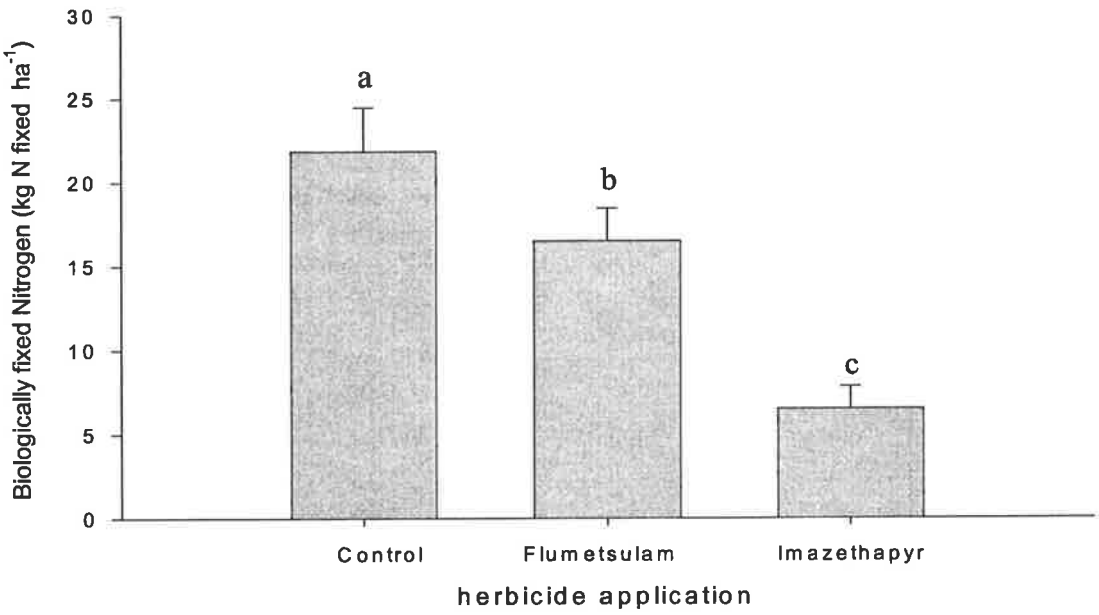


Figure 5.14: Additional herbicide (flumetsulam and imazethapyr) effects on biologically fixed nitrogen of chickpea plants. Bars indicate standard error of mean. Similar letters indicate significant similarity as determined by Tukey's H.S.D. test.

When biologically fixed nitrogen per hectare was converted to nitrogen fixed per unit of shoot biomass, significant additional herbicide effects were observed (Table 5.7). The imazethapyr treatment had a significantly lower biologically fixed nitrogen than the no additional herbicide and flumetsulam treatments with a 38% reduction in the amount of nitrogen fixed per unit of shoot biomass (Figure 5.15).

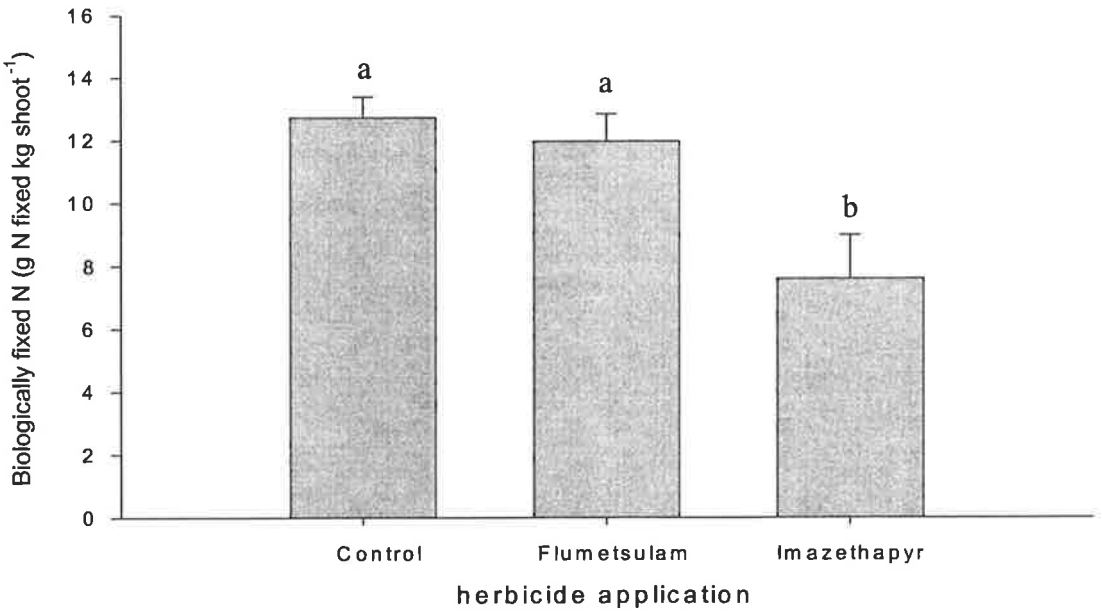


Figure 5.15: Herbicide (flumetsulam and imazethapyr) effects on biologically fixed nitrogen per unit of shoot biomass. Bars indicate standard error of mean. Similar letters indicate significant similarity as determined by Tukey's H.S.D. test.

5.3.6 Nutrient analysis

Nutrient analysis was undertaken to ensure that nutrients were not limiting and therefore only herbicide effects were being observed. The mean results for major nutrients showing adequate levels for chickpeas and analysis of variance data are shown in Table 5.8. All nutrients show adequate levels except for copper and zinc which are both lower than the average adequate levels recorded for chickpeas in the literature (Table 5.8) (Reuter and Robinson, 1997). However, these are consistently low across all treatments.

Table 5.8: Major nutrients of *M. rugosa* shoots at flowering showing chlorsulfuron, flumetsulam and spinnaker treatments. Adequate, marginal (marg.) or critical deficiency (c.d) of a similar species, *M. truncatula* are shown in bold for each nutrient. Standard deviations are shown in parentheses. Results of analysis of variance are shown with significant results shown in bold.

Herbicide	Chlorsulfuron (%)	P 2400 c.d.	K 10000-36000	S 1500 c.d	Ca 13000-22000	Mg 1500 marg.	Cu 4-35 c.d	Zn 12-500	Mn 60-300	B 22-30
Nil	0	2200 (535)	23500 (2645.8)	1665 (243.4)	21550 (3348.1)	2675 (309.6)	3.3 (0.5)	12.2 (1.9)	46.426.6 (19.5)	34 (7.0)
Nil	5	2450 (264.6)	22750 (957.4)	1805 (73.3)	23500 (3696.8)	2800 (163.3)	3.3 (0.3)	13.7 (1.8)	53.2 (12.7)	40.7 (2.8)
Nil	10	2525 (170.8)	21850 (3496.2)	1600 (372.6)	20475 (2963.5)	2775 (520)	3.6 (0.5)	14.1 (1.1)	49.8 (12.7)	35.9 (1.9)
Nil	20	2350 (57.7)	19700 (3453.5)	1685 (37)	20150 (6113.1)	2700 (391.6)	3.2 (0.4)	12.5 (0.9)	45.7 (13.4)	33.1 (6.3)
Flumetsulam	0	2525 (377.5)	23000 (2160.2)	1700 (84.5)	24500 (4795.8)	2725 (189.3)	3.3 (0.2)	14.9 (2.3)	50.7 (7.8)	38.4 (4.2)
Flumetsulam	5	2525 (250)	20900 (1604.2)	1625 (60.3)	23750 (1500)	2575 (50)	3.3 (0.3)	14.5 (1.8)	50.9 (11.6)	37.1 (4.5)
Flumetsulam	10	2350 (369.7)	20300 (4275.5)	1642.5 (234.1)	20750 (957.4)	2725 (221.7)	3.3 (0.6)	13.1 (2.1)	46.1 (9.3)	35.3 (2.4)
Flumetsulam	20	2225 (95.7)	18325 (4009.5)	1587.5 (94.6)	19700 (424.3)	2625 (170.8)	3.2 (0.7)	13.1 (0.5)	43.8 (2.4)	35.6 (2.1)
Imazethapyr	0	2775 (543.9)	21500 (1000)	1850 (177.6)	22175 (3304.9)	3150 (506.6)	3.9 (0.6)	14.4 (3.1)	55.4 (18.8)	42.8 (7.5)
Imazethapyr	5	2750 (387.3)	22250 (2500)	1907.5 (131.5)	20300 (1174.7)	3150 (525.9)	3.9 (0.7)	14.5 (2.2)	51.1 (15.1)	40.5 (9.5)
Imazethapyr	10	2407.5 (520.5)	19525 (1231.2)	1752.5 (135)	19600 (5168.5)	2850 (387.3)	3.2 (0.5)	12.0 (2.5)	42.7 (15.5)	37.5 (10.1)
Imazethapyr	20	2525 (457.3)	17675 (1703.7)	1757.5 (264.1)	17500 (3207)	2900 (355.9)	3.6 (0.4)	13.2 (1.3)	43.9 (4.4)	38.3 (4.3)
P value										
Chlorsulfuron		0.288	**0.015**	0.397	0.085	0.522	0.792	0.397	0.267	0.350
Herbicide		0.324	0.263	0.097	0.097	0.166	0.291	0.432	0.934	0.202
Chlorsulfuron*herbicide		0.288	0.805	0.761	0.795	0.230	**0.023**	0.220	0.555	0.469

5.3.7 Rainfall data

The monthly rainfall distribution for 1997, and long term average from 1957 – 1997) are shown in Table 5.9. Late rains fell in September, October, November and December. The total annual rainfall was 442.2 mm. The highest monthly rainfall was in September, followed by October and May. The months of June and July had below average rainfalls, whilst the months of September, October and November had above average rainfalls.

Table 5.9: Monthly rainfall distribution for 1997 in paddock west 7 at Roseworthy Campus of University of Adelaide.

Month	Rainfall (mm)	Long term average rainfall (mm) for Roseworthy (1957 – 1997)
January	26.4	19.7
February	38.6	18.6
March	4.0	18.6
April	10.8	33.5
May	51.6	52.3
June	24.8	41.8
July	14.8	53.9
August	39.4	52.2
September	92.0	48.3
October	60.4	41.1
November	43.4	24.9
December	36	24.9
Total	442.2	429.8

5.4 DISCUSSION

At the time of flowering (October), the dry matter production of chickpeas not treated with herbicides in this field trial, was within the world wide reported range of dry matter production in desi type chickpea cultivars of 1550 - 8200 kg ha^{-1} (Armstrong *et al.*, 1997; Horn *et al.*, 1996a; Kurdali, 1996; Siddique *et al.*, 1993; Beech and Leach, 1989; French and Ewing, 1989). Apart from differences in varieties, the large range in dry matter production reported is due to differences in time of sowing, soil type, tillage practices and climatic conditions (Armstrong *et al.*, 1997; Horn *et al.*, 1996a; Kurdali, 1996; Siddique *et al.*, 1993; Beech and Leach, 1989; French and Ewing, 1989). During the growing season in this field trial, observations of stunted growth were made in all herbicide treatments, suggesting that the herbicides were affecting shoot growth and plant health. Imazethapyr and high residual rates of chlorsulfuron both had negative affects on dry matter production of chickpeas. Chickpeas grown in the presence of residual concentrations of chlorsulfuron, had negative growth rates at the time of flowering, suggesting that the plants were beginning to senesce. Chlorsulfuron is an inhibitor of cell division and growth in sensitive species (Ray, 1982a), leaving the plant in a state of stress by flowering, probably due to an inability to take up nutrients and moisture from the soil.

Jettner *et al.* (1999), reported that 0.94 ug ai L^{-1} (equivalent to 10% of the recommended field application rate) of chlorsulfuron induced 50% inhibition of desi chickpea (cv Barwon) seedling growth, 28 days after sowing in a soil free growth medium. In a soil free growth medium, the herbicide would be fully available as the confounding effect of soil adsorption onto soil particles would be eliminated and all the

herbicide would remain in solution (Jettner *et al.*, 1999). Therefore, the potential damage to the crops based on application rate may have been overestimated (Jettner *et al.*, 1999). In the field trial discussed in this chapter, chlorsulfuron at 10% of the recommended field application rate, was found to inhibit shoot growth and leaf area index and this inhibition increased over the growing season. A greater inhibition was observed when chlorsulfuron was applied at 20% rather than 10% of the recommended application rate. The observed reductions in growth were similar to the results of Jettner *et al.* (1999) and establish a trend of chlorsulfuron residues reducing growth of chickpeas.

Leaf area index (LAI) is a measure of the photosynthetic area over a given area of ground (Lawrence, 1989) and is an important determinant of plant productivity (Khanna Chopra and Lakshmi, 1987). Crop growth rate generally increases with increasing leaf area index, but only until the canopy intercepts all incident light (Shibles and Weber, 1965). Mckenzie *et al.* (1992), found that an increased leaf area index of Kabuli type chickpea resulted in an increase in intercepted solar radiation, which in turn increased dry matter production. Leaf area and dry matter production are very slow in chickpeas for a long time after sowing (until flowering is initiated) (Khanna Chopra and Sinha, 1987). For example, a field study in India found that the LAI of JG 62, a desi variety of chickpea, was less than 1 by the time of flowering (Aggrawal *et al.*, 1984) and this resembles the LAI of the control treatments from this field trial which were approximately 0.8 – 0.9 at flowering. The lower LAI's from imazethapyr or high residual levels of chlorsulfuron, may partly explain the reduction in dry matter production and growth rates observed in these treatments, as less light was intercepted leading to less dry matter production.

The lowest yields of chickpea from the field trial discussed in this chapter, were found in the highest chlorsulfuron treatments consistent with the data on shoot biomass, leaf area index and growth rates. Low yields were also observed in the control treatment, with no residual or additional herbicide application. This result may partly be explained by the observed presence of weeds in this treatment and the unusual rainfall pattern at the end of the growing season. Chickpeas are poor competitors with weeds because of their slow winter growth (Siddique and Pritchard, 1993) and the herbicides in other treatments may have effectively removed competition. Thus, the removal of competition may account for imazethapyr in the nil and 5% chlorsulfuron treatments showing higher yields than the control treatments with no residual or additional herbicide, although this is not supported by the results for shoot biomass, nitrogen and growth rates. During the growing season, observations in the field identified restrictions in growth and much yellowing of plants in the imazethapyr treatments. However, the 1997 season was characterised by an initial dry period with significant late rain in November and December. At the time of late rain, the control and flumetsulam treatments had begun to form pods and senesce. However, the late rain gave imazethapyr treated plants a chance to recover and form pods later (3 – 4 weeks) than other treatments. These plants senesced much later and delayed the harvest until January. It was therefore possible that the yield from imazethapyr plots was influenced and improved by the late rain. A study in India, reported that the period of fruit development and the rate of leaf senescence in chickpea was related to the onset of water stress and increasing temperatures (Khanna-Chopra and Sinha, 1987). Rain will cause the pods to develop and mature over a longer period, and the leaves senesce at a slower pace. Conversely, lack of rain coupled with a sudden rise in temperature can result in premature senescence of the crop (Khanna-Chopra and Sinha, 1987).

Flumetsulam (20 g ai ha $^{-1}$) or imazethapyr (72g ai ha $^{-1}$) applied 4-7 months before sowing had no effect on the yield of chickpeas grown in southern Queensland (Barnes *et al.,* 1996). The application rate of imazethapyr in the study by Barnes *et al.* (1996) was a higher application rate than in this field trial but support the observation that imazethapyr had little effect on the yield of chickpeas, except in combination with the highest simulated residual rate of chlorsulfuron. Another study in South Australia reported that flumetsulam (25g ha^{-1}) application slightly increased chickpea yield by 8% compared to nil herbicide application (Wheeler *et al.*, 1996), and this may have been due to reduced competition. These results support the results from the field trial discussed in this chapter, where flumetsulam application increased yield probably due to enhanced weed control.

The literature shows that the seed yields of desi chickpeas in Australia vary from 160 kg ha^{-1} to 2900 kg ha^{-1} (Jettner *et al.*, 1999; Armstrong *et al.*, 1997; Horn *et al.*, 1996a; Siddique *et al.*, 1993). The lowest yields measured in the field trial discussed in this chapter were found in imazethapyr + 20% chlorsulfuron and flumetsulam + 20% chlorsulfuron treatments, and were below the range of chickpea yields reported in the Australian literature, suggesting that these herbicide combinations had a negative effect on chickpea yield. The combination of 20% recommended application rate of chlorsulfuron and flumetsulam or imazethapyr, applied 'in-crop', may have resulted in an inhibition of cell division and growth (mode of action of ALS–inhibiting herbicides) leading to an inability to form pods. Variations in yield arise for the same reasons discussed earlier for dry matter production, and include different varieties, sowing time, soil type, tillage practices and climatic conditions (Armstrong *et al.*,1997; Horn *et al.*,

1996a; Kurdali, 1996; Siddique *et al.*, 1993; Beech and Leach, 1989; French and Ewing, 1989).

In the absence of herbicides, the proportion of nitrogen fixed by chickpea plants in this trial was 52% and was within the range of 8 - 82% reported for Australia (Peoples *et al.,* 1995b). The amount of nitrogen biologically fixed by chickpeas in this field trial, decreased with increasing rates of chlorsulfuron and from the control to flumetsulam and imazethapyr. This trend reflected the herbicide impacts on shoot biomass. As both total plant nitrogen and biologically fixed nitrogen (kg N fixed ha^{-1}) were calculated using shoot biomass, it is possible that the observed effects were due to a reduction in shoot biomass rather than any specific reduction in nitrogen. Because of this biologically fixed nitrogen was converted to kg N fixed/tonne shoot matter and therefore everything should be comparable on a unit basis. The %Ndfa of chickpeas in this field trial also decreased when the combination of high residual levels of chlorsulfuron and flumetsulam or imazethapyr were present, suggesting that these herbicide affected nitrogen fixation. Pre-emergent applications of imazethapyr have been shown to reduce nitrogenase activity (measured by acetylene reduction activity, ARA) of pea and soybeans grown in Poland by 85% and 60% respectively (Sawicka *et al.*, 1996). Post-emergent applications of imazethapyr reduced nitrogenase activity of peas, yellow lupins, white lupins and soybeans grown in Poland by approximately 15, 50, 55 and 87% respectively (Sawicka *et al.*, 1996). The ARA method for measuring nitrogen fixation is now considered unreliable because it provides only an instantaneous measure of nitrogenase activity, without taking into account diurnal and seasonal changes (Shearer and Kohl, 1986). However, the results of Sawicka *et al.* (1996) do

support those from the current field trial where imazethapyr reduced biologically fixed nitrogen of chickpeas by approximately 40%.

In Sweden, chlorsulfuron applied at 2 g ha^{-1} reduced nitrogenase activity (ARA) of lucerne by approximately 50% when compared to a control treatment (Martensson and Nilsson, 1989). This reduction in nitrogenase activity was attributed to adverse effects of the herbicide on plant growth and not on rhizobia (Martensson and Nilsson, 1989). Biologically fixed nitrogen in the field trial discussed in this chapter was also reduced by chlorsulfuron at this rate, and at a slightly higher rate of 20% of the recommended application rate. This reduction was probably caused by an impact on shoot biomass, more than a direct effect on nitrogen fixed, as the effect was removed after expressing the amount of nitrogen fixed as a function of shoot biomass. Other studies have investigated the effects non ALS-inhibiting herbicides on nitrogenase activity of legumes as measured by ARA and are summarised in Table 5.9. The results from all of these trials, including the current field trial, suggested that some herbicides reduced the amount of nitrogen fixed. This does not always correspond with a reduction in yield but, because of the importance of nitrogen fixation in farming systems (Unkovich *et al.*, 1997; Vance *et al.*, 1997; Giller and Cadisch, 1995; Peoples *et al.*, 1995b) it is necessary to be cautious when using these herbicides. The reduction in nitrogen fixation due to herbicide use, could lead to reduced input of nitrogen to the soil, and thus increased fertiliser costs for the farmer (Eberbach, 1993). In contrast, some herbicides including EPTC, benefin, profluralin and diclofop have been shown to have little effect on nitrogen fixation of lucerne as measured by ARA (Peters and Zbiba, 1979). If these herbicides control weeds effectively in legume crops they may provide a better alternative to some of the other herbicides discussed.

Most of the major nutrients examined in this study had values that were above adequate levels reported in the literature for chickpeas (Reuter and Robinson, 1997). Copper and manganese levels were low (Reuter and Robinson, 1997) across all treatments suggesting that observed herbicide effects were due to the herbicides and not nutrient limitations.

Table 5.9 The effects of herbicides on nitrogen fixation of legumes as measured by acetylene reduction activity (ARA).

Plant	Herbicide	ARA reduction (%)	Reference
Soybean	Alachlor	32	Bollich *et al.*, 1985
			Mallik & Tesfai, 1985.,
	Metribuzin	50	Bertholet & Clark, 1985
			Bollich *et al.*, 1985
			Mallik & Tesfai, 1985
	Trifluralin	Reduction	Bollich *et al.*, 1988
			Mallik & Tesfai, 1985
	Prometryn	32	Bollich *et al.*, 1985
	Linuron	37.5	Rennie & Dubetz, 1984
	Pendimethalin	Reduction	Bollich *et al.*, 1988
Faba beans	Metribuzin	92	Bertholet & Clark, 1985
Lupins	Simazine	40	De Felipe *et al.*, 1987
	Lindex	40	De Felipe *et al.*, 1987
White clover	Paraquat		Rolston *et al.*, 1976
Red clover	Dinoseb	Reduction	Lindstrom *et al.*, 1985
Subterranean clover	Amitrole	Reduction	Eberbach & Douglas, 1991
	Diquat	Reduction	Eberbach & Douglas, 1991
	Trifluralin	Reduction	Eberbach & Douglas, 1991

5.5 SUMMARY AND KEY FINDINGS

Overall, the results showed that high residual levels of chlorsulfuron and imazethapyr reduced chickpea shoot biomass, nitrogen fixation and to a lesser extent yield. Imazethapyr alone and in combination with low rates of chlorsulfuron did not

reduce chickpea yield, although the results may have been influenced by climatic conditions late in the season. Yield was much more responsive to rainfall in the post flowering period. Chickpea yield was reduced by the combination of high residual levels of chlorsulfuron and imazethapyr. The combination of high residual levels of chlorsulfuron and imazethapyr could occur in farming rotations, if chlorsulfuron was applied to cereal crops in one year and imazethapyr was used to control weeds in a subsequent legume rotation. Black *et al.* (1999) recovered 5% and 17% of applied chlorsulfuron (11ng g^{-1}) 24 months after application at two sites in south-eastern Australia. The 5% recovery reported by Black *et al.* (1999) approximates the 5% residual level used in the field trial in this chapter. It is therefore possible that the lower residual rates of chlorsulfuron (5% recommended application rate) used in this field trial may still remain in the soil 24 months after application. However, these lower residual rates did not reduce shoot biomass or yield to the same extent as the higher residual rates. In soils with a pH of between 7.5 and 8.5, the recommended plant back period is 24 months (Chambers, 1995) and the results of this field trial suggest that herbicide effects may still be evident after 12 months. The results of this field trial and that of Black *et al.* (1999) suggest that the plant back period of 24 months should be observed as a minimum.

Chlorsulfuron and imazethapyr both reduced biologically fixed nitrogen of chickpeas. The chlorsulfuron effects on nitrogen fixation are probably due more to adverse effects on shoot biomass, than directly on fixation. Imazethapyr did reduce nitrogen fixation. This has implications for the nitrogen balance of the soil for the following crop and as such the use of this herbicide may reduce the benefits of using a

grain legume in a cropping sequence. In addition, it could increase farmer costs due the need for nitrogenous fertilisers.

The results have shown that nitrogen fixation of chickpeas was reduced by chlorsulfuron and imazethapyr. This reduction in nitrogen fixation may be due to a direct herbicide effect on the plant, the herbicide affecting nodulation, or the herbicide affecting rhizobia. The following chapters will investigate these possibilities in further detail beginning with the effects on nodulation. Chapter 6 will establish the experimental protocol for a pot trial to investigate the impacts of chlorsulfuron and imazethapyr on nodulation of chickpeas.

CHAPTER 6

DEVELOPMENT OF METHODOLOGIES FOR AN EXPERIMENT INVESTIGATING THE EFFECTS OF ALS-INHIBITING HERBICIDES ON NODULATION OF CHICKPEA

6.1 INTRODUCTION

Experimental results presented in Chapter 5 demonstrated that chlorsulfuron and imazethapyr reduced growth and nitrogen fixation of chickpea plants. However, the mechanisms by which the herbicides are reducing growth and nitrogen fixation are unclear. In order to understand the impact these herbicides have on the symbiosis between chickpea plants and their associated *Rhizobium* (CC1192), an experimental approach has been designed to investigate the impacts that these herbicides may have on the rate of formation and number of N fixing root nodules. This is best undertaken in a pot trial using controlled environmental conditions.

To conduct an experiment investigating the effects of these herbicides on nodulation of chickpeas the following design criteria were considered necessary:

1) Plants grown in a sterilised growth medium to ensure that there was no contamination of the pots with other rhizobia or microorganisms;

2) A pH of at least 8.0 in the growth medium is required as the herbicides persist under alkaline conditions, and inhibition of growth in the field (Chapter 5 and other studies) was observed at high pH;

3) Maintenance of a virtually nitrogen free environment during these experiments was necessary to ensure that all nitrogen, except that in the seed, was derived from symbiotic nitrogen fixation. Nitrogen is known to inhibit nodulation and nitrogen fixation of legumes (Cowie *et al.*, 1990; Ewing and Robson, 1990; Butler, 1988; Streeter, 1988).

The experiments in this chapter were used to define the experimental conditions under which chickpea plants would be grown in subsequent work aimed at defining the impact of the herbicides on the nitrogen fixation process. It was necessary to ensure that growth conditions during the experiment were adequate so that herbicide treatment effects could be attributed to the addition of the herbicide rather than to other experimental factors such as pot size, growth medium or nutrient solution.

The aims of the experiments in this chapter were:

1. To define a growth medium in which to conduct the experiment ensuring that the plants grow adequately;

2. To determine the most appropriate nutrient solution to use for watering the plants ensuring that all nutrients are in adequate supply and plant growth would not be limited;

3. To determine the most appropriate pot size to use for the experiment, ensuring that plants do not become pot bound and growth is not inhibited within the planned time frames of the experiments.

The results will be used to determine experimental protocols for the two future studies described in Chapters 7 and 8.

6.2 METHODS AND MATERIALS

6.2.1 Selection of growth media

Two separate experiments were conducted. The first compared the growth of chickpea plants in vermiculite to chickpea growth in a mixture of 50% vermiculite and 50% sand (by volume). The second experiment compared the 50% vermiculite and 50% sand mixture to pure sand. The mixture will be referred to as vermiculite sand mixture throughout the chapter. Vermiculite and sand are often used in experiments for *Medicago* and *Trifolium* species (Vincent, 1970) and it was decided to trial these media and the vermiculite sand mixture for chickpeas. Both experiments were established as described below.

The pots used in this experiment were 250 ml tissue culture containers with lids to prevent microbial contamination of the growth media. The lids had a breather hole to facilitate watering. The breather hole was stoppered with a pop rivet to prevent contamination (see Plate 7.1 in Chapter 7). An extra hole (7mm diameter) was drilled in each lid for seedling placement.

Before planting the seeds, the pots were filled with washed vermiculite (washed as vermiculite is often very alkaline with pH 11) or the vermiculite sand mixture for the first experiment, and either sand or the vermiculite sand mixture for the second experiment. The pots were then saturated with 1/4 strength Jensen's solution (Vincent, 1970) which is a nitrogen deficient nutrient solution often used for the growth of legumes (1.0 g $CaHPO_4$ L^{-1}, 0.2 g K_2HPO_4 L^{-1}, 0.2 g $MgSO_4.7H_2O$ L^{-1}, 0.2 g NaCl L^{-1}, 0.1 g $FeCl_3$ L^{-1}; and 1 ml trace element solution (2.86 g H_3BO_3 L^{-1}; 2.08 g L^{-1} $MnSO_4.4H_2O$; 0.222 g L^{-1} $ZnSO_4.7H_2O$; 0.079 g L^{-1} $CuSO_4.5H_2O$ and 0.09 g L^{-1}

$H_2MoO_4.H_2O$) and made up to 1 litre with distilled water). The lids were placed on the pots and the pots were then sterilised by autoclaving at 121°C for 30 minutes. After removal from the autoclave the holes were covered with ethanol soaked parafilm to help keep the contents sterile until the seedlings were ready for planting.

Chickpea seeds were surface sterilised to remove any microbial contaminants by the following method of Vincent (1970):

1. Rinsed with ethanol for one minute and then decanted,
2. Rinsed with dilute hypochlorite for one minute and then decanted,
3. Rinsed with sterile deionised water 8 times and left to stand for 15 minutes,
4. Rinsed with sterile deionised water a further 8 times and left to imbibe for three hours,
5. The water was decanted after three hours and seeds were spread aseptically on sterile 2% water agar in petri dishes. Petri dishes were wrapped in plastic wrap to avoid dehydration and then placed in an incubator at 25°C for 48 hours for germination.

All plates were free from bacterial and fungal growth at the end of the 48 hour period. The germinated seedlings were aseptically transferred into the pre-drilled holes in the lids of the 250 ml pots using sterile forceps. The seed sat just on the surface of the lid, with the radicle protruding into the vermiculite. Seedlings were then covered with tissues, and saturated with sterile deionised water to keep moist until the seedling roots had become established in the growth media. The seedlings were then covered with plastic wrap and placed in the glasshouse for two days. After two days the plastic

wrap was removed. At this time, each pot was inoculated with 1 ml of chickpea *Rhizobium* (CC1192) containing 1 x 10^9 cells ml^{-1} that had been cultured in yeast mannitol broth (0.5 g K_2HPO_4 L^{-1}; 0.2 g $MgSO_4.7H_2O$ L^{-1}; 0.1 g NaCl L^{-1}; 10.0 g mannitol ($C_6H_{14}O_8$) L^{-1}; 1.0 g yeast L^{-1}) (Vincent, 1970) for 48 hours at 25°C on an shake incubator.

Each treatment was replicated four times and the pots were arranged in a randomised design in the glasshouse. Plants were watered weekly with 1/4 strength Jensen's solution and harvested at 4 weeks. At this time root and shoot samples were dried at 60°C for 48 hours to obtain dry weights. Results were analysed by analysis of variance using Genstat release 4.1 (Payne, 1993).

6.2.2 Determination of most appropriate nutrient solution

Jensen's solution at 1/4 and 1/8 strength and McKnight's solution at full and 1/2 strength were compared to determine which provided the best plant growth. Jensen's solution is described in section 6.2.1. McKnight's solution (according to Bergersen, 1980) consisted of 0.14 g $FeCl_3$ L^{-1}; 0.2 g KH_2PO_4 L^{-1}; 1.5 g $CaSO_4.2H_2O$ L^{-1}; 0.2 g $MgSO_4.7H_2O$ L^{-1}; 0.3 g KCl L^{-1} and 1 ml of a trace element solution (2.86 g H_3BO_3 L^{-1}; 0.222 g $ZnSO_4.7H_2O$ L^{-1}; 0.079 g $CuSO_4.5H_2O$ L^{-1} and 0.09 g $H_2MoO_4.H_2O$ L^{-1}). The pH of all nutrient solutions were adjusted to pH 8.0 with NaOH to approximate the soil pH levels at which impacts on the growth of chickpea plants were observed following herbicide application in previous field experiments. By watering with a nutrient solution of pH 8.0, it was possible to maintain pH at this level throughout the experiment and pH was measured at the end of the experiment to ensure this.

Seedling, *Rhizobium* inoculation and general pot preparation used were the same as those described in section 6.2.1 with the exception that only vermiculite was used as the growth media in this experiment. The four nutrient solutions described above were used in place of the 1/4 Jensen's solution referred to in section 6.2.1.

The plants were grown for four weeks and watered weekly (40% water holding capacity) during this time with their respective nutrient solutions. There were four replicates of each treatment which were placed in a randomised design in a controlled environment room, with day time temperatures of 21°C and night time of 16°C. The daylength was 14 hours. At four weeks, the plants were harvested and dry weights for roots and shoots were taken. Significant (@ α=0.05) results were determined by analysis of variance using the program Genstat Release 4.1 (Payne, 1993). Only significant results will be presented.

6.2.3 Selection of appropriate pot size

Two experiments were conducted in order to select the optimum pot size for the subsequent experiments. The first compared the 250 ml tissue culture containers, that were used in the previous trials discussed in this chapter, to 500 ml tissue culture containers. These larger tissue culture containers were similar to the 250 ml pots in that they had breather holes and extra holes were drilled in the lids to allow for seedling placement. A further comparison was performed between the 500 ml pots (with lids) and larger more conventional pots of 2500 ml capacity, as plants becoming root bound in the tissue culture containers during the experiment was a concern. It was not possible to sterilise the 2500 ml pots. Other than not sterilising the 2500 ml pots, the experiment was set up using the same design described in section 6.2.1 in terms of seedling and *Rhizobium* inoculant preparation.

In this experiment, the 250 ml and 500 ml pots were covered in aluminium foil to keep light from reaching the roots (Plate 7.1, Chapter 7). As the larger 2500 ml pots were black, this covering was not necessary. The plants were grown in pure sand and watered to 40% gravimetric water holding capacity with 1/2 strength McKnight's solution when necessary.

The first experiment, comparing the 250 ml and 500 ml pots, was harvested at 4 weeks with the number of nodules and shoot and root dry weight determined. The second experiment, comparing the 500 ml and 2500 ml pots, was harvested at 2 and 4 weeks and samples were taken for root and shoot dry biomass. Significant results were determined by analysis of variance using Genstat Release 4.1 (Payne, 1993). Only significant results will be presented.

6.3 RESULTS

6.3.1 Selection of growth media

There was no significant difference between the shoot biomass of chickpea plants grown in vermiculite and those grown in the vermiculite sand mixture ($p=0.455$).

There was a significant difference between the root biomass of chickpea plants grown in vermiculite and those grown in the vermiculite sand mixture ($p=0.034$). Chickpea plants grown in the vermiculite sand mixture had 36% greater root biomass (0.458g $plant^{-1}$) than those grown in pure vermiculite (0.294 g $plant^{-1}$).

There was no significant difference in chickpea shoot biomass ($p=0.310$) or root biomass ($p=0.605$) in plants grown in sand or in the vermiculite sand mixture.

6.3.2 Determination of most appropriate nutrient solution

Chickpea shoot biomass ($p=0.496$) and root biomass ($p=0.830$) did not differ significantly between 1/4 or 1/8 strength Jensen's or, full or 1/2 strength McKnight's nutrient solutions.

6.3.3 Selection of appropriate pot size

There was a significant difference in shoot biomass when comparing 250 ml and 500 ml pot sizes ($p<0.001$). Shoot biomass was 30% greater in the larger 500 ml pots (Table 6.1). The root biomass of chickpea plants also differed significantly between 250 ml and 500 ml pots ($p<0.001$), with root biomass 40% greater in the 500 ml pots (Table 6.1). There was no significant difference in the number of nodules found on chickpea plants grown in either the 250 ml or 500 ml pots ($p=0.384$).

Table 6.1: Significant effects of different sized pots on root and shoot biomass of chickpeas $\pm$ standard error of mean.

	250 ml	500 ml
Root biomass (g)	0.352 ± 0.0217	0.5896 ± 0.0352
Shoot biomass (g)	0.2635 ± 0.0123	0.3700 ± 0.0113

Whilst there was no significant difference between shoot biomass of plants grown in 500 ml and 2500 ml pots ($p=0.326$), there was a significant interaction between harvest time and pot size on root biomass ($p=0.030$). There was little difference in root biomass between the two pot sizes over the first two harvests, but at the final harvest, root biomass was 20% greater in the 500 ml pot than the 2500 ml pot (Figure 6.1).

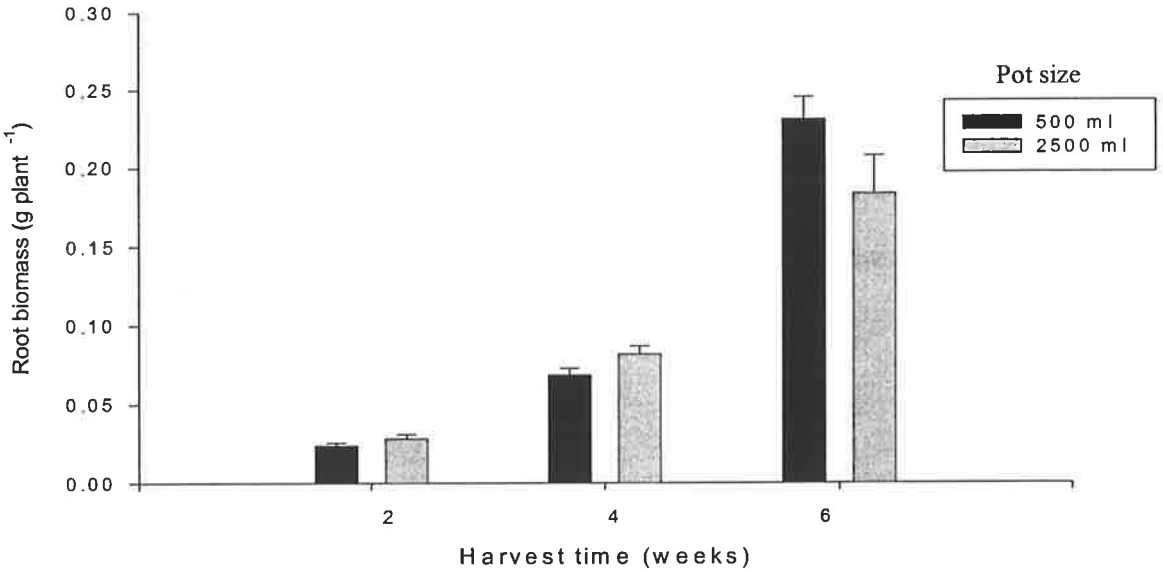


Figure 6.1: Root biomass of chickpea plants grown in 250 ml and 500 ml pots containing pure sand and harvested at 2,4 and 6 weeks. Bars indicate standard error of mean.

6.4 DISCUSSION AND CHOICE OF EXPERIMENTAL PROTOCOLS

Chickpea root biomass was greater when grown in a mixture of vermiculite and sand compared to a pure vermiculite medium. However, there was no difference in shoot or root biomass when comparing the vermiculite sand mixture and pure sand. As the experimental design of future experiments will involve large numbers of pots and therefore large volumes of growth media, the mixture of sand and vermiculite was considered unsuitable due to the difficulty in ensuring an even mix of both vermiculite and sand. Although a coarse sand was used in these trials, a finer sand was selected for future experiments in order to increase the water holding capacity of the growth media. This finer sand was used in the earlier experiment discussed in Chapter 3.

Chickpea plant biomass was not affected by the various nutrient solutions. McKnight's 1/2 strength solution was chosen as the nutrient solution for future experiments. As plants will be grown in an enclosed system, continuous watering with full strength nutrient solution may result in the development of nutrient toxicities.

Therefore the 1/2 strength McKnight's solution was chosen for the experiments presented in chapters 7 and 8. Jensen's solution was not selected because of potential sodium toxicity originating from its sodium content and sodium added while adjusting the growth media pH with NaOH.

Chickpea plant biomass was greatest when grown in 500 ml pots with lids. This pot size was chosen for the future experiments because they provided a greater volume for the plant roots than the 250 ml pots, they were autoclavable, and the lids aided in maintaining sterile conditions over the duration of the future experiment.

During these trials, it was observed that some seedlings desiccated early and never recovered. To overcome this problem, the seedlings were pre-germinated on agar plates for 72 hours rather than 48 hours stated in section 6.2.1 to allow the radicles to grow to a greater length. When planting the seedlings, small holes were made in the sand using a sterile probe, into which the radicle was placed. This allowed planting without breaking the radicle and also ensured adequate contact between the radicle and growth media. In addition, the seedlings were left covered with moistened tissues for three days to ensure adequate moisture until the plant was capable of extracting sufficient water from the sand. An extra hole was also drilled into each lid, to allow two seedlings to be inserted into each pot. After one week the seedlings were thinned to one per pot. After removing the seedling, the vacated hole was covered for sterility purposes.

6.5 SUMMARY

Results from the experiments discussed in this chapter have defined the protocol required to investigate the effects of herbicides on chickpea growth and nodulation

under controlled environment conditions. Subsequent experiments designed to investigate the effects of the herbicides, chlorsulfuron (Chapter 7) and imazethapyr (Chapter 8) on nodulation of chickpea plants will use the following protocol:

1. The plants will be:
    - grown in 500 ml pots covered with aluminium foil (see Plate 7.1);
    - in fine sand (lucerne medic mix –described in chapter 7);
    - watered with 1/2 strength McKnight's solution adjusted to pH 8.0 with NaOH.
2. The seeds will be germinated on agar for 72 hours.
3. Two seedlings will be planted in each pot and:
    - inoculated with rhizobia (grown in YMB adjusted to pH 8.0 using NaOH) at the time of planting;
    - covered with moistened tissues after planting, for 72 hours;
    - placed in a controlled environment room (25°C day and 16°C night), 14 h daylight and light intensity of approximately 900 μmol m^{-2} s^{-1};
    - thinned to one pot^{-1} after one week in controlled environment room.

CHAPTER 7

THE EFFECTS OF CHLORSULFURON ON CHICKPEA GROWTH, NODULATION AND *RHIZOBIUM*

7.1 INTRODUCTION

It was shown in Chapter 5 that the ALS-inhibiting herbicide, chlorsulfuron, reduced the growth of chickpea and may inhibit nitrogen fixation in the field. The formation of nodules in the legume – *Rhizobium* symbiosis is necessary for symbiotic nitrogen fixation, and symbiotic nitrogen fixation contributes directly to the productivity of forage and grain legumes (Sprent and Sprent, 1990). The infection of plant roots by *Rhizobium* and the subsequent formation of nodules is complex. Details of the root infection process were presented in section 2.6.2 of Chapter 2.

Little work has investigated the effects of ALS-inhibiting herbicides on the nodule formation process. Chlorsulfuron inhibited either early root hair infection or the number of nodules formed by *Medicago sativa* (Koopman *et al.*, 1995; Martensson, 1992; Martensson and Nilsson, 1989); sub clover (Eberbach and Douglas, 1989) and red clover (Martensson, 1992). These results suggest that ALS-inhibiting herbicides inhibited the nodule formation of the legumes studied, even though rhizobial populations were not necessarily affected. Inhibition of nodulation may have had a deleterious effect on nitrogen fixation and hence may have influenced soil nitrogen fertility.

To date, no studies have investigated the effects of chlorsulfuron residues on chickpea *Rhizobium*, nodulation and nitrogen fixation. This chapter reports the results of an experiment to determine if chlorsulfuron affects the ability of chickpea *Rhizobium* to infect the host plant roots, and subsequently affect nodule formation.

The aim of the experiment was to investigate the effects of chlorsulfuron on the ability of chickpea *Rhizobium* to effectively nodulate chickpea.

7.2 MATERIALS AND METHODS

7.2.1 Treatments and experimental set up

Chickpea *Rhizobium* (CC1192) were grown as described in Chapter 6, section 6.2 and 6.5. The rhizobia used in the experiment were grown in the presence or absence of filter sterilised (0.2 μm) chlorsulfuron (see Table 7.1 for application rates). An uninoculated control to check for contamination was included. The application rate of chlorsulfuron was representative of exposure levels that natural rhizobia populations would experience in soil, where chlorsulfuron was applied at the recommended field application rate, assuming 100% bioavailability. After treatment for 48 hours, rhizobia were centrifuged at 5000 rpm for 10 minutes then rinsed and re-centrifuged three times in ¼ strength Ringers solution (2.25 g L^{-1} NaCl; 0.105 g L^{-1} KCl; 0.12 g L^{-1} $CaCl_2.6H_2O$; 0.05 g L^{-1} $NaHCO_3$; pH adjusted to 8.0 with NaOH) in order to remove any herbicide adhering to the rhizobial cells. After the third rinse, the rhizobia were re-suspended in phosphate buffer (78.0 g L^{-1} $NaH_2PO_4.2H_2O$; 179.25 g L^{-1} $Na_2HPO_4.12H_2O$) prior to inoculation.

Chickpea seeds were pre-germinated in the presence, or absence, of chlorsulfuron. Prior to germination, seeds were surface sterilised as described in Chapter 6. Filter sterilised (0.2 μm) herbicides were applied to the agar plates before adding the sterilised seeds. Chlorsulfuron was added at 10% of the recommended field application rate to represent residual bioavailability in an alkaline soil (Table 7.1). After three days, seedlings with radicles of similar length were selected for planting into each pot.

The chickpea plants were grown in 500 ml polycarbonate plant tissue culture containers described in Chapter 6. The pots were filled with a potting mix recommended for growing legumes such as lucerne or medic. The potting mix (soil) consisted of coarse white and grey sands. The potting mix was the same as that used in Chapter 3, except that no fertilisers were added and the soil nitrogen content was approximately 0.01%. This potting mix will be referred to as 'soil' throughout Chapters 7 and 8. During the experiment, the pots were kept at 60% water filled pore space (total porosity) for optimum plant growth. This was calculated by determining the bulk density of the soil (equation 7.1) and calculating total porosity (equation 7.2):

$$\text{Bulk density (BD)} = \frac{\text{mass of soil}}{\text{volume of container}} \qquad (7.1)$$

$$\text{Total porosity (TP)} = \frac{\text{BD}}{\text{PD}} \qquad (7.2)$$

Where PD is the particle density and was assumed to be 2.65.

Pots were then maintained at 60% water filled pore space.

Initially, 50 ml of ½ strength Mcknight's solution were added to each pot to wet the soil for autoclaving, as described in Chapter 6. A nitrogen treatment was also

included in the experiment and comprised 0.072g L^{-1} of NH_4NO_3 added to ½ strength McKnight's solution as described above. The added nitrogen treatment will be referred to as nitrogen fertiliser throughout Chapters 7 and 8 to distinguish it from fixed and seed nitrogen.

The pots with moistened soil were autoclaved at 121°C for 30 minutes with autoclave tape placed over the breather holes to maintain sterility until the seedlings were ready for planting. Prior to planting the seedlings, the soil was adjusted to 60% water filled pore space, using sterile ½ strength McKnight's nutrient solution either with, or without, nitrogen. Chlorsulfuron was also added to the nutrient solution to ensure even distribution throughout the pot (Table 7.1). Two seedlings were placed into each pot as described in Chapter 6 and 1 ml of the appropriate rhizobia inoculum was added as close to the radicle as possible. The pots were placed in a controlled environment room with 25°C maximum and 16°C minimum temperature and light intensity of approximately 900 μmol m^{-2} s^{-1}. Seedlings were thinned to one per pot after one week in the controlled environment room.

Table 7.1: Chlorsulfuron application rates at each stage of experimental set up (rec. app. rate = recommended application rate).

Stage	Chlorsulfuron application	
Rhizobia	0.01 μg ai ml^{-1}	Rec. app. rate
Germination	0.001 μg ai ml^{-1}	10% rec. app. rate.
Soil/pot	0.001 μg ai ml^{-1}	10% rec. app. rate.

7.2.2 Experimental design

The experiment was set up as a randomised block design with each of five treatment replicates arranged in separate blocks. Treatments were applied in a five factor factorial design as follows:

- 3 x rhizobia treatments (presence or absence of herbicide; uninoculated control).

  Rhizobia were grown in the presence, or absence, of the herbicide to determine any herbicide effects on their ability to infect plant roots. The uninoculated control was included to ensure there was no rhizobial contamination.

- 2 x germination treatments (presence or absence of herbicide).

  Chickpea seeds were pre-germinated on agar in the presence, or absence, of the herbicide to investigate any herbicide effects on germination and whether these carried over to affect nodulation.

- 2 x soil treatments (presence or absence of herbicide).

  Chickpea plants were grown in potting mix in the presence, or absence, of the herbicide to determine herbicide effects on plant growth and nodulation.

- 2 x nitrogen treatments (presence or absence of fertiliser nitrogen).

  A nitrogen treatment was included to ensure that inoculated plants were growing well and to investigate whether the addition of nitrogen fertiliser could help the plants overcome any reductions in growth.

- 2 x harvest (three and six weeks after inoculation).

  Two harvests were undertaken to determine if herbicide effects on plant growth and nodulation change over time and establish herbicide effects on growth rate.

7.2.3 Sampling and Measurements

Chickpea plants were harvested three and six weeks after inoculation to quantify treatment effects on growth rate. Measurements taken are summarised in Table 7.2.

Total shoot area of chickpeas was estimated using a scanner (HP Scanjet 6100, calibrated and tested by Regent Instruments) and the WinSeedle program, and then chickpea shoots and roots were dried at 60°C for 48 hours before weighing for biomass. Total root length was measured using a scanner (as above) with the WinRhizo program and then converted to root length density by dividing by the volume of the pots in which the plants were grown.

Absolute growth rates (AGR) were calculated by using equation 7.3:

$$AGR = \frac{\text{Shoot dry weight at time 2} - \text{Shoot dry weight at time 1}}{\text{Time 2(days)} - \text{time 1 (days)}} \quad (7.3)$$

Relative growth rates (RGR) were calculated by using equation 7.4:

$$RGR = \frac{\text{ln shoot dry weight at time 2} - \text{ln shoot dry weight at time 1}}{\text{Time 2 (days)} - \text{time 1 (days)}} \quad (7..4)$$

The number of nodules per plant were counted after hand washing the roots. As the nodules were generally small at the first harvest, fresh weight of nodules was only measured at the second harvest. The nodules were picked from the roots using forceps, then placed on tissues to remove excess moisture before weighing. The weight of nodules refers to total nodule weight per plant.

Dried chickpea roots and shoots were ground using a mortar and pestle (due to low biomass from single plants) and were analysed for total nitrogen using a CN-2000 LECO carbon/nitrogen analyser. Root and shoot nitrogen were determined separately, however, results were combined, to determine total plant nitrogen. An estimate, or inference, of the amount of nitrogen fixed by the chickpea plants could be made for

plants grown without the addition of nitrogen fertiliser. Seed nitrogen was analysed as previously described, and was then subtracted from the combined root and shoot plant nitrogen. The total plant nitrogen of the uninoculated control plants was also subtracted from the plant nitrogen of the inoculated plants to account for the approximately 0.01% nitrogen in the soil (potting mix). In this way it was possible to get an estimate, or inference, of the amount of nitrogen fixed using equation 7.5.

N fixed = (Total N of inoculated – seed N) – (total N of uninoculated – seed N) (7.5).

Table 7.2: Summary of samples taken at three and six week harvests for chlorsulfuron experiment (4 indicates that samples were taken).

Parameter	3 weeks	6 weeks
Shoot biomass	4	4
Shoot area		4
Root biomass	4	4
Root length	4	4
Nodule number	4	4
Nodule fresh weight		4
Total nitrogen	4	4
Nitrogen fixed (inference)		4

7.2.4 Data interpretation

Significant (@ α=0.05) results were determined by analysis of variance that included main effects of rhizobia, germination, soil, nitrogen, and harvest time, and their interactions, using Genstat 5 Release 4.1 (Payne, 1993). 'Rhizobia' refer to pre-exposure and non pre-exposure of the rhizobia to chlorsulfuron. On the graphs, + , – , or 0 rhizobia represent pre-exposed, non-pre-exposed and non-inoculated rhizobia treatments respectively. 'Germination' effects refer to pre-germinating chickpea seeds in the presence (+), or absence (-) of chlorsulfuron. 'Soil' effects are those observed as

a result of the presence (+) or absence (-) of herbicide in the soil (potting mix). 'Nitrogen' effects refer to the presence (+) or absence (-) of nitrogen fertiliser (i.e. nutrient solution containing nitrogen). 'Harvest' effects are effects over time from the harvests at three and six weeks and are referred to as harvest 1 or 2. Graphs of significant treatment effects and interactions show means obtained from an average of all other treatments.

Interpretation of the data was complex due to four way interactions. In this situation, the data were split at one level and another analysis of variance was performed on the two separate data sets. For example: in a four way interaction, with harvest as one level, the data was split into harvests one and two, and then analysed again.

The uninoculated treatments were included as controls to check for contamination and were expected to have no nodules. Consequently, the data for nodule counts and fresh weight were skewed and the analysis of variance was invalid. To accommodate this, the nodule counts and weights were treated as a difference from the uninoculated treatments, which removed the zero values from the data set and allowed valid analysis of variance.

Linear regression analyses to determine the relationship between nodule weight and nodule number, and nodule weight and plant nitrogen content, were performed using Sigma Plot for Windows Version 5.00 (SPSS Inc).

7.3 RESULTS

7.3.1 Shoot biomass and total shoot area

The reduction in shoot biomass of chickpea plants grown with chlorsulfuron present during rhizobial growth, seed germination and in the soil compared to plants grown in the absence of chlorsulfuron at each stage, is shown in Plate 7.1. Shoot weight was significantly affected by two 3 way interactions (Table 7.3). There was a significant interaction between the presence of chlorsulfuron in the soil, nitrogen fertiliser and harvest (Table 7.3; Figure 7.1). There was no difference between treatments at the first harvest (Figure 7.1). After six weeks of growth, the presence of chlorsulfuron in the soil reduced chickpea shoot biomass (Figure 7.1). The addition of nitrogen fertiliser increased shoot biomass by 23% in the absence of chlorsulfuron and decreased shoot biomass by 17% in the presence of chlorsulfuron (Figure 7.1).

Plate 7.1: A comparison of chickpea plants with chlorsulfuron present during rhizobial growth, seed germination and in the soil (right) and those grown in the absence of chlorsulfuron (left), six weeks after inoculation. The plants were grown in a controlled environment room (25°C/16°C temperatures; 14 hours daylight), in 500 ml tissue culture containers with lids containing a breather hole. The breather hole was stoppered with a pop rivet to aid in maintaining sterility. The pots were covered with aluminium foil to prevent light reaching the roots.

Table 7.3: The significant (α=0.05) effects of chlorsulfuron applied at different stages on shoot biomass and shoot area of chickpea plants.

Variable	Source	P-value
Shoot weight	Soil*nitrogen*harvest	0.014
	Rhizobia*nitrogen*harvest	0.027
	Rhizobia*germination	0.007
Shoot area	Rhizobia*germination*soil*nitrogen	0.017
Shoot area (- nitrogen)	Rhizobia*germination*soil	0.049
Shoot area (+ nitrogen)	Soil	<0.001

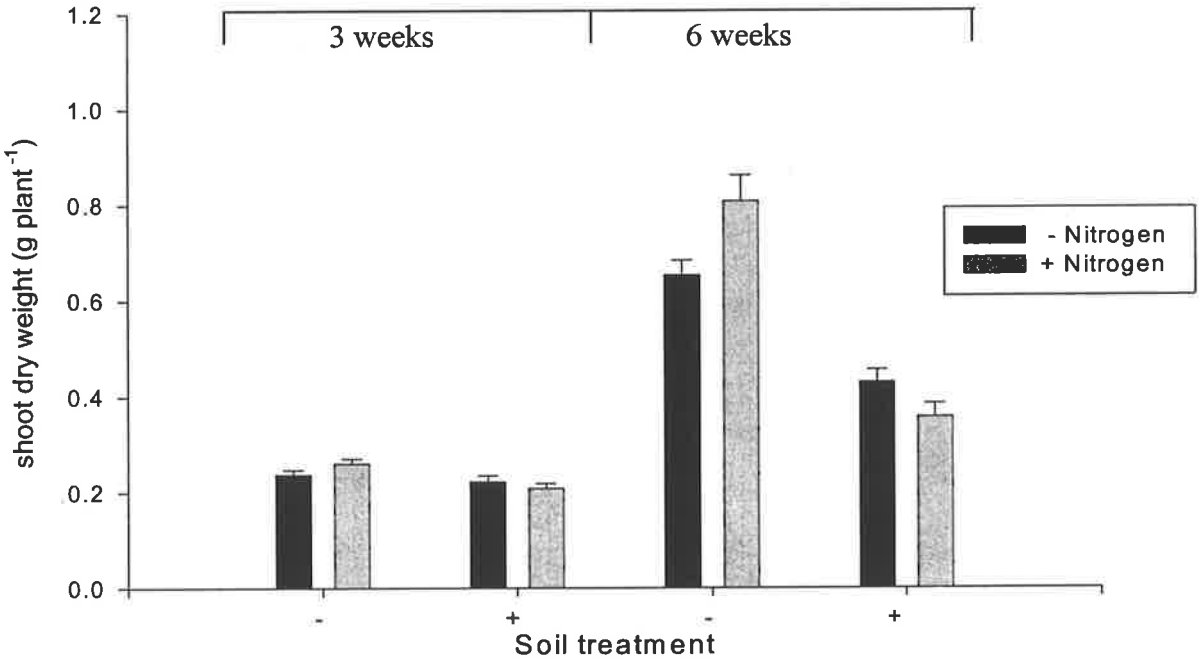


Figure 7.1: The effects of presence (+) and absence (-) of chlorsulfuron in the soil, nitrogen fertiliser (presence(+) /absence (-)) and harvest time (three and six weeks) on shoot biomass of chickpeas grown in a controlled environment room. Bars indicate standard error of mean.

Shoot biomass of chickpeas was also affected by an interaction between rhizobia, nitrogen and harvest (Table 7.3). There was no difference between treatments at the first harvest (Figure 7.2). Shoot biomass increased with the addition of nitrogen in both pre-exposed and uninoculated rhizobia treatments at the second harvest (Figure 7.2).

Pre-exposure of rhizobia and pre-germinating seeds in the presence of chlorsulfuron interacted to affect shoot biomass (Table 7.3). In the pre-exposed rhizobia and uninoculated treatments, shoot biomass was greater in those plants that had been pre-germinated in the presence of the chlorsulfuron compared to those that had not (Figure 7.3). However, when the rhizobia were not pre-exposed, the shoot biomass was greater when the seeds were pre-germinated without chlorsulfuron (Figure 7.3).

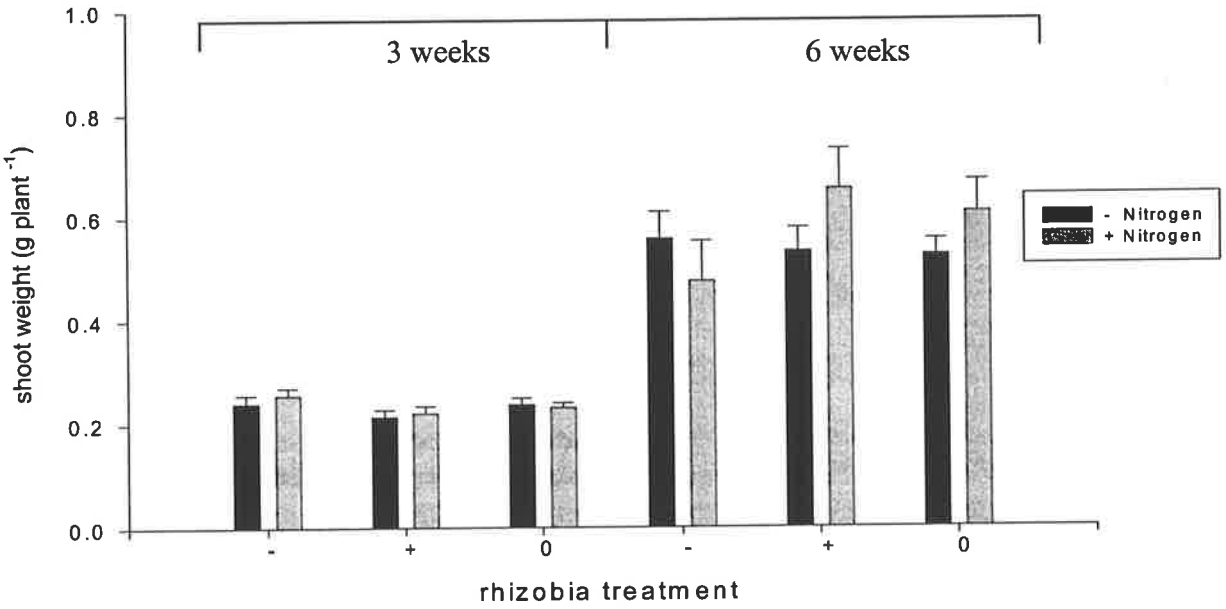


Figure 7.2: The effects of rhizobia treatment with chlorsulfuron (pre-exposed (+), not pre-exposed (-) and uninoculated (0)), nitrogen fertiliser (presence (+)/absence (-)) and harvest (3 and 6 weeks) on shoot biomass of chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

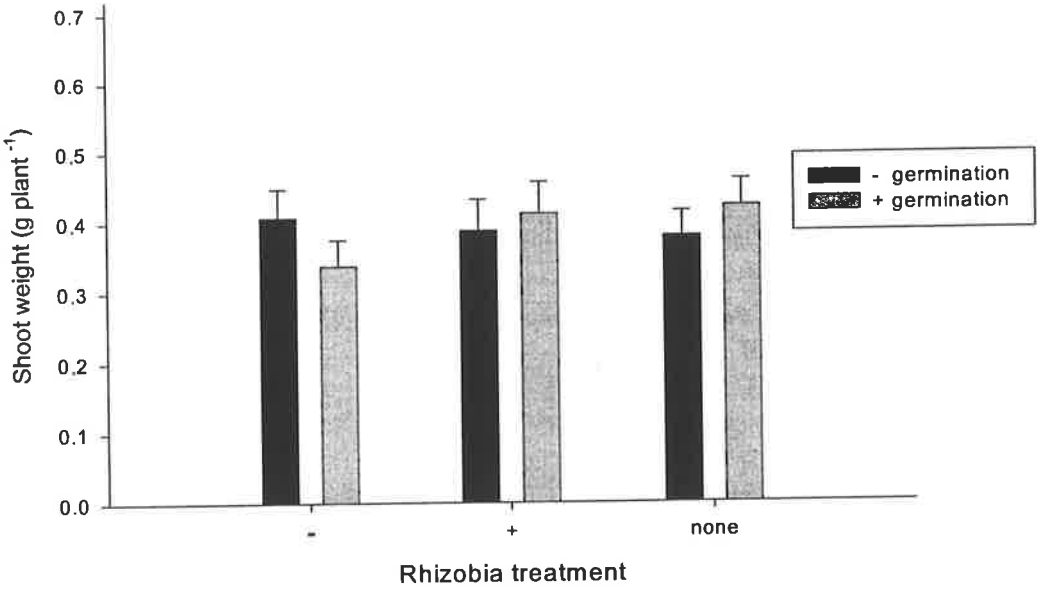


Figure 7.3: Effects of rhizobia and germination treatments on shoot biomass of chickpea plants. The rhizobia treatments were: not pre-exposed (-) and pre-exposed (+) to chlorsulfuron and uninoculated (none). The germination treatments were: pre-germinated in presence (+) and absence (-) of chlorsulfuron. Bars indicate standard error of mean.

Shoot area of chickpea plants at six weeks after inoculation was affected by a four way interaction between rhizobia, germination, presence of chlorsulfuron in the soil and nitrogen fertiliser (Table 7.3; Figure 7.4). Because the four way interaction was complex to describe, the data were split at the nitrogen level, and + and - nitrogen treatments were analysed separately (Table 7.3). The - nitrogen fertiliser data showed a significant three way interaction between rhizobia, germination and soil (Table 7.3; Figure 7.4). The presence of chlorsulfuron in the soil reduced shoot biomass of chickpeas regardless of other treatments (Figure 7.4). When chlorsulfuron was absent from the soil, and plants were inoculated with non-pre-exposed rhizobia, germinating seeds in the presence of chlorsulfuron reduced shoot area by 26% compared to those germinated without chlorsulfuron (Figure 7.4). However, when chlorsulfuron was present in the soil, and plants were inoculated with pre-exposed rhizobia, germinating seeds in the presence of chlorsulfuron resulted in a 100% increase in shoot area,

compared to plants germinated without the herbicide (Figure 7.4). Plants inoculated with pre-exposed rhizobia, in the absence of chlorsulfuron in the soil and at germination, had a reduced shoot area of 59% compared to those inoculated with non-pre-exposed rhizobia (Figure 7.4). When chlorsulfuron was present in the soil, non-pre-exposed rhizobia treatments had greater shoot area when seeds were germinated in the presence of chlorsulfuron, compared to those germinated in the absence of chlorsulfuron (Figure 7.5). Pre-exposed rhizobia treatments, however, had lower shoot area when seeds were germinated in the presence of chlorsulfuron compared to those germinated in the absence (Figure 7.4).

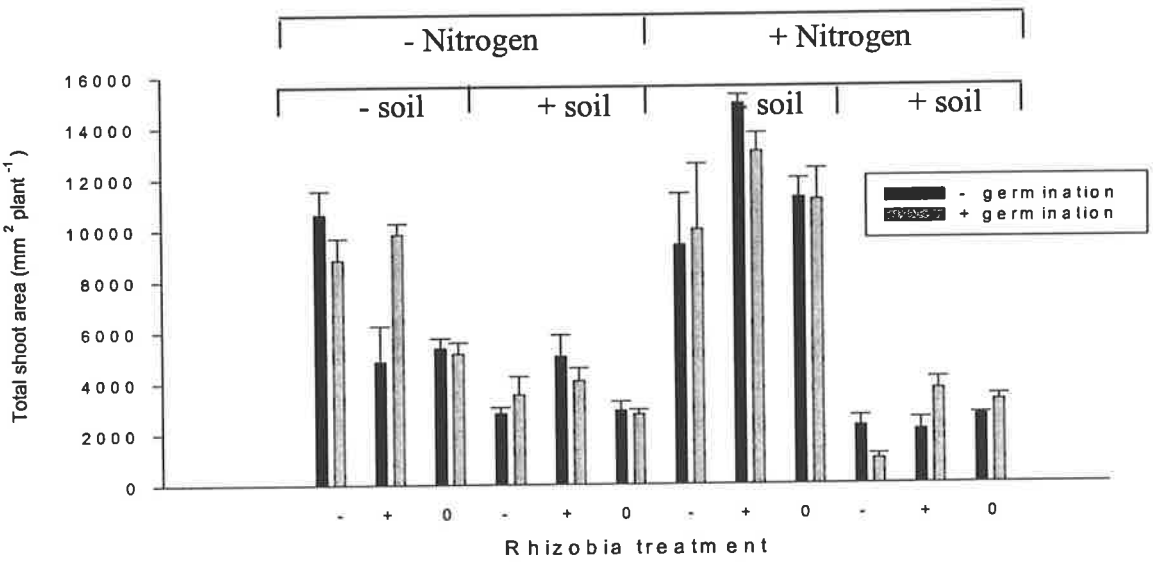


Figure 7.4: Effects of rhizobia, germination, chlorsulfuron in the soil and nitrogen (+/-) on total area of chickpea shoots six weeks after inoculation grown in a controlled environment room. Rhizobia treatments (x-axis) were non pre-exposed (-) and pre-exposed (+) to chlorsulfuron and uninoculated (0). Germination treatments (legend) were germinated in the presence (+) and absence (-) of chlorsulfuron. Soil treatments were presence (+) and absence (-) of chlorsulfuron in the soil. Bars indicate standard error of mean.

The + nitrogen data had a significant soil main effect (Table 7.3). The presence of chlorsulfuron in the soil resulted in a 78% (11680 mm^2 compared to 2564 mm^2) reduction in the shoot area of chickpea plants.

7.3.2 Root biomass and root length density

Table 7.4 shows significant results for chickpea root biomass and root length density as determined by the analysis of variance. Chickpea root biomass was affected by an interaction between the presence of chlorsulfuron in soil, nitrogen fertiliser and harvest (Table 7.4; Figure 7.5). Root biomass increased from the first to the second harvest (Figure 7.5). The presence of chlorsulfuron in the soil reduced chickpea root biomass at both harvests, but the reduction was greater at the second harvest, at six weeks (Figure 7.5). At the second harvest, the addition of nitrogen fertiliser increased the biomass of chickpea roots by 20% in soils without chlorsulfuron (Figure 7.5). When nitrogen fertiliser was added to soils with chlorsulfuron, the root biomass was reduced by 40% in comparison to those plants grown without additional nitrogen (Figure 7.5).

Table 7.4: Significant (α=0.05) effects of chlorsulfuron applied at different stages on biomass and root length density of chickpea roots.* = interaction

Variable	Source	P value
Root biomass	Soil*nitrogen*harvest	0.033
	Rhizobia*nitrogen*harvest	0.021
Root density (both harvests)	Rhizobia*soil*nitrogen*harvest	0.022
	Rhizobia*germination	0.035
Root density (harvest 1)	Soil*nitrogen	0.001
Root density (harvest 2)	Rhizobia*soil*nitrogen	0.034

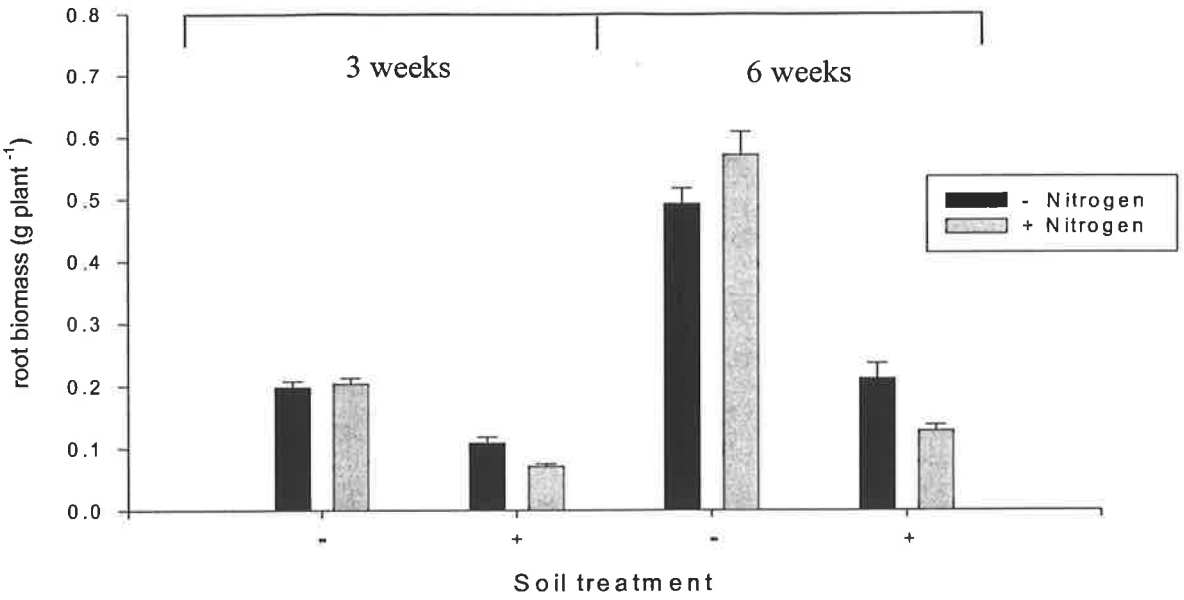


Figure 7.5: Effects of the presence and absence chlorsulfuron in soil, nitrogen fertiliser (presence (+)/absence (-)) and harvest (three and six weeks) on root biomass of chickpea plants grown in a controlled environment room. Bars indicate standard error.

Chickpea root biomass was also influenced by an interaction between rhizobia treatments, nitrogen fertiliser and harvest (Table 7.4). At the second harvest, the biomass was greater than at the first, and each of the rhizobia treatments varied in their response to nitrogen (Figure 7.6). At the second harvest, chickpea root biomass of non pre-exposed rhizobia treatments was 25% lower when nitrogen fertiliser was present, compared to those grown without nitrogen fertiliser. The addition of nitrogen fertiliser increased root biomass by 19% and 15% respectively in plants that were not inoculated, or were inoculated with rhizobia that had been pre-exposed to chlorsulfuron (Figure 7.6). At the first harvest there was little difference between the treatments, with the exception of a decrease in root biomass, when nitrogen was added to the uninoculated treatment (Figure 7.6).

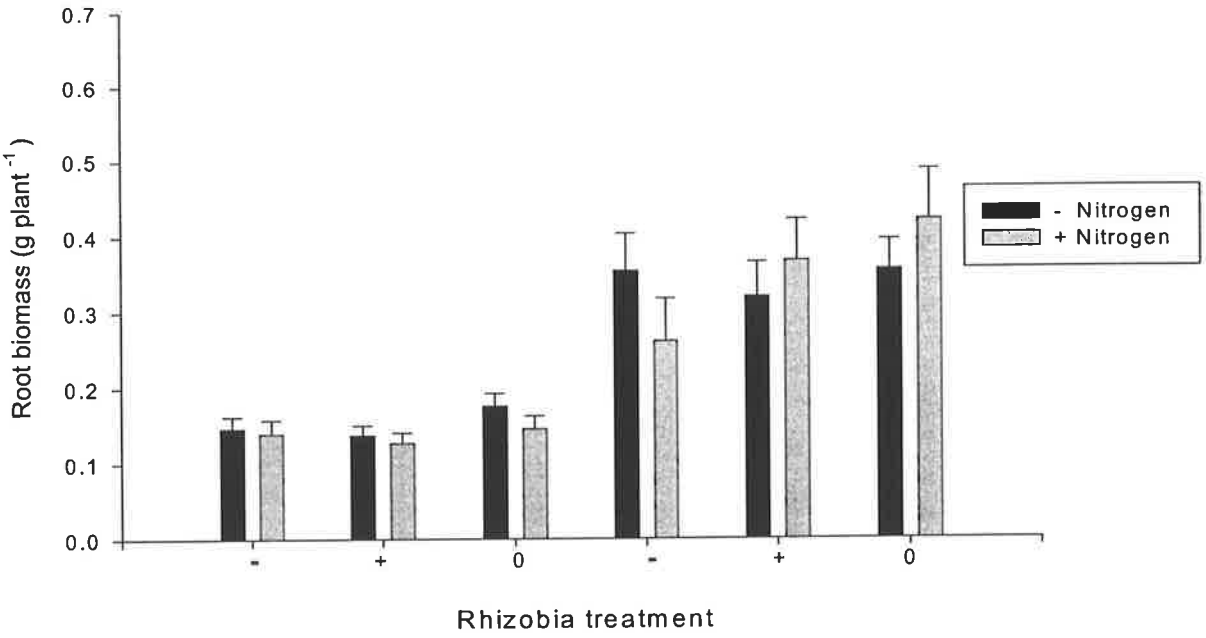


Figure 7.6: Effects of rhizobia (not pre-exposed (-), pre-exposed (+) uninoculated (0)), nitrogen fertiliser (presence (+)/absence(-)) and harvest (3 and 6 weeks) on root biomass of chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

Root length per unit volume of soil, or root length density, of chickpeas was significantly affected by a four way interaction between rhizobia treatments, presence of chlorsulfuron in the soil, nitrogen fertiliser and harvest (Table 7.4; Figure 7.7). The data were split on the basis of harvest and re-analysed for separate harvests (Table 7.4). A significant soil and nitrogen interaction was found at the first harvest and a significant rhizobia, soil, and nitrogen interaction was observed at the second harvest (Table 7.4).

After three weeks of growth, the root length density of chickpeas was reduced by 56% in the presence of chlorsulfuron in the soil and absence of nitrogen fertiliser (Figure 7.8). The addition of nitrogen fertiliser increased root length density by 10% when chlorsulfuron was absent from the soil, but decreased root length density by 46% in the presence of chlorsulfuron (Figure 7.8).

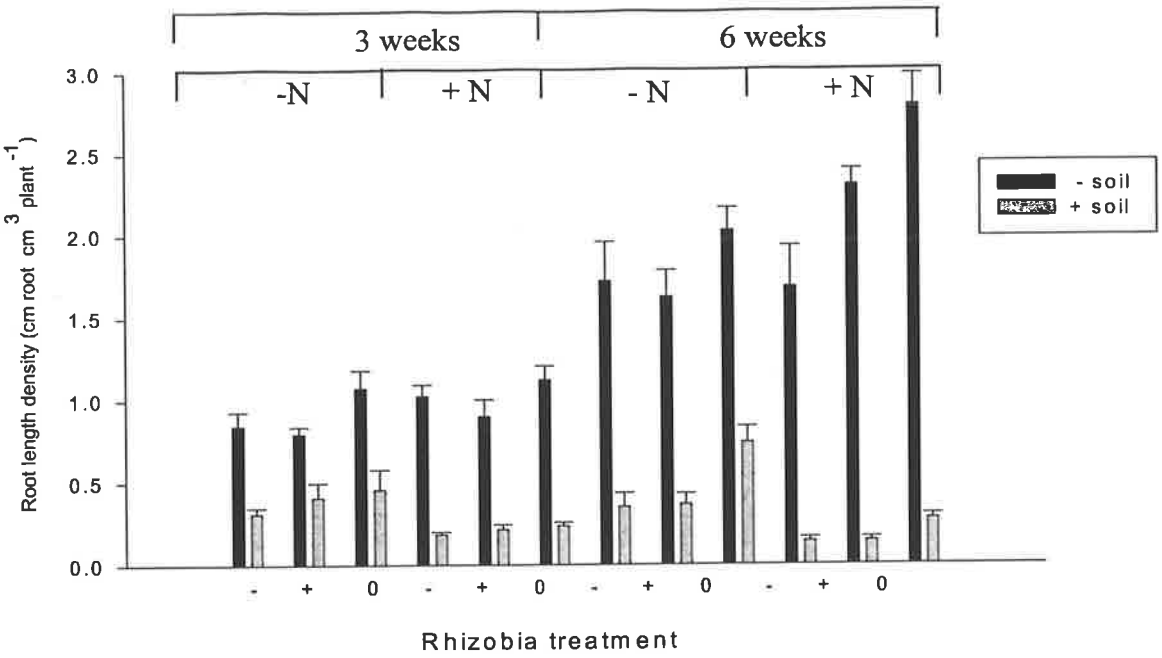


Figure 7.7: Effects of rhizobia treatments (non pre-exposed (-), pre-exposed (+) uninoculated (0)), chlorsulfuron in the soil, nitrogen fertiliser (+/-) and harvest (3 and 6 weeks) on root length density of chickpea plants. +/- N, rhizobia and soil indicate presence/absence of nitrogen, and chlorsulfuron during rhizobial growth and in the soil respectively. Bars indicate standard error of mean.

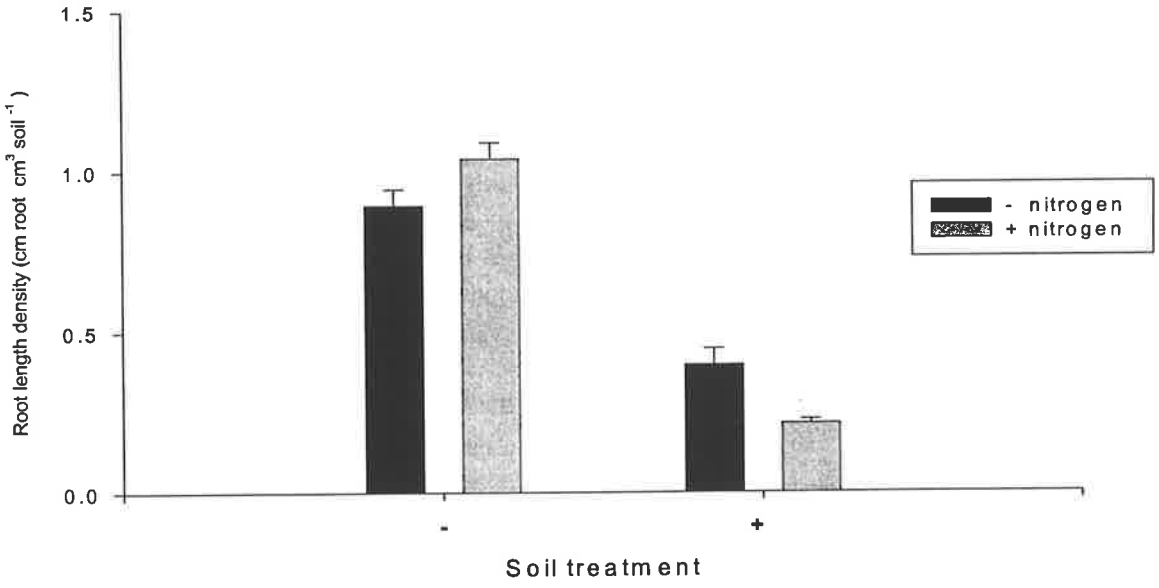


Figure 7.8: Effects of presence and absence of chlorsulfuron in the soil and nitrogen fertiliser (+/-) on root length density of chickpeas at harvest one, three weeks after inoculation. Bars indicate standard error of mean.

After six weeks of growth, root length density was influenced by an interaction between rhizobia treatments, presence of chlorsulfuron in the soil and nitrogen fertiliser (Table 7.4). When chlorsulfuron was present in the soil, the root length density of chickpeas was reduced and the addition of nitrogen fertiliser led to a further reduction in root length density across all rhizobia treatments (Figure 7.9). Rhizobia treatments differed in their response to nitrogen fertiliser. When chlorsulfuron was absent from the soil, the addition of nitrogen fertiliser increased root length density of plants that were either inoculated with pre-exposed rhizobia or not inoculated (Figure 7.9). Whilst those that were inoculated with non pre-exposed rhizobia had similar root length densities with and without the addition of nitrogen (Figure 7.9).

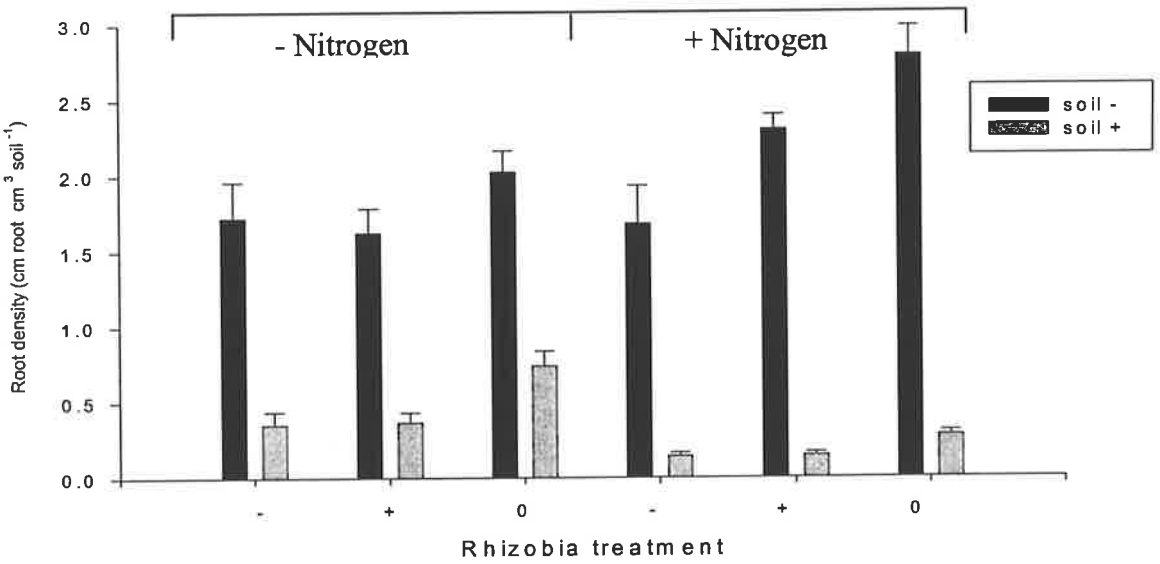


Figure 7.9: Effects of rhizobia (not pre-exposed (-) and pre-exposed (+) to chlorsulfuron and uninoculated), presence (+) and absence (-) of chlorsulfuron in the soil and nitrogen fertiliser (presence (+)/ absence(-)) on root length density of chickpea plants six weeks after inoculation. Bars indicate standard error of mean.

Root length density of chickpeas (from both harvests combined) was also affected by an interaction between rhizobia and germination treatments in addition to

the four way interaction previously described (Table 7.4). In the plants inoculated with non-pre-exposed rhizobia, root length density was reduced by 23% when seeds were pre-germinated in the presence of chlorsulfuron, compared to those pre-germinated in the absence of chlorsulfuron (Figure 7.10). The opposite was true for plants that were inoculated with pre-exposed rhizobia, where root length densities increased by 10% when the seeds were pre-germinated in the presence of chlorsulfuron (Figure 7.10).

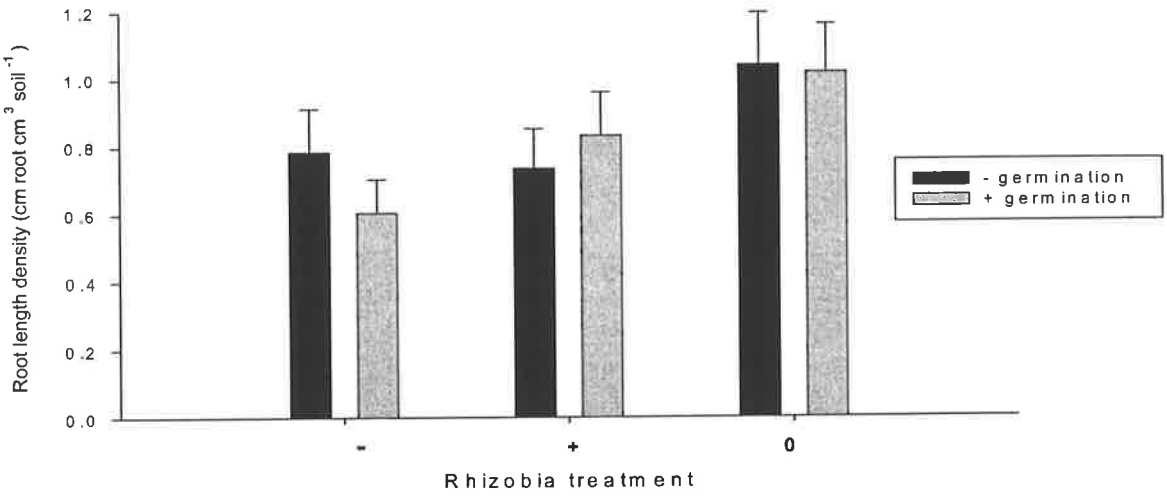


Figure 7.10: Effects of rhizobia (non pre-exposed (-), pre-exposed (+), uninoculated (0) and pre-germinating seeds in the presence (+) and absence (-) of chlorsulfuron root length density of chickpeas grown in a controlled environment room. Bars indicate standard error of mean.

7.3.3 Number and weight of nodules

The number of nodules found on each chickpea plant was significantly affected by a three way interaction between rhizobia and germination treatments and the presence of chlorsulfuron in the soil (Table 7.5). Rhizobia treatments responded differently to the presence of chlorsulfuron at germination and the presence of chlorsulfuron in the soil (Figure 7.11). The number of nodules decreased when chlorsulfuron was present in the soil, regardless of rhizobia or germination treatments.

However, when chlorsulfuron was absent from the soil, pre-exposure of rhizobia to chlorsulfuron reduced nodule numbers by approximately 51% when compared to those that were not pre-exposed (Figure 7.11). The reduction in nodule number due to pre-exposure of rhizobia to chlorsulfuron was observed when chlorsulfuron was absent both at germination and in the soil. When chlorsulfuron was present at germination and in the soil, pre-exposing rhizobia increased the number of nodules by 24% (Figure 7.11).

Table 7.5: Significant (α=0.05) effects of chlorsulfuron on number and weight of nodules as determined by analysis of variance.

Variable	Source	P value
Number of nodules	Soil*harvest	<0.001
	Rhizobia*germination*soil	0.010
Nodule weight	Rhizobia *nitrogen	0.034
	Rhizobia * germination	<0.001
	Soil	0.031

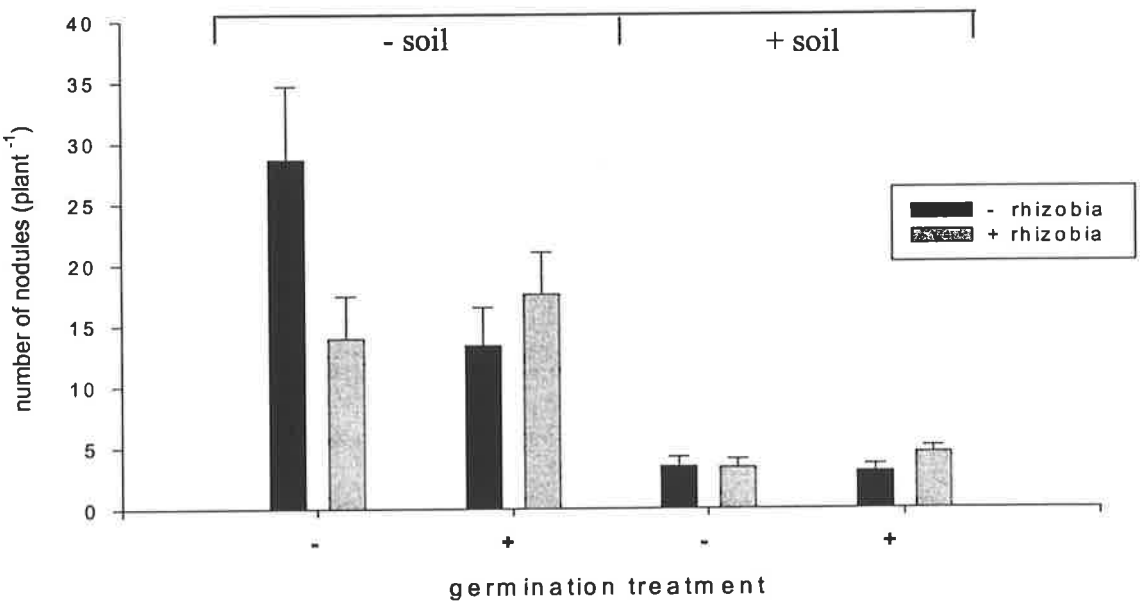


Figure 7.11: Effects of rhizobia (not pre-exposed (-), pre-exposed (+)), germination of seeds (in the presence and absence of chlorsulfuron) and the presence and absence of chlorsulfuron in the soil on the number of nodules formed on chickpea plants. Bars indicate standard error of mean.

An interaction between harvest time and the presence of chlorsulfuron in the soil also affected the nodule count of chickpea plants (Table 7.5). At both the three and six week harvest, the presence of chlorsulfuron in the soil reduced the number of nodules on chickpea roots (Figure 7.12). The reduction in the number of nodules, due to the presence of chlorsulfuron in the soil, was greater at the second harvest (90%) than the first (47%) (Figure 7.12).

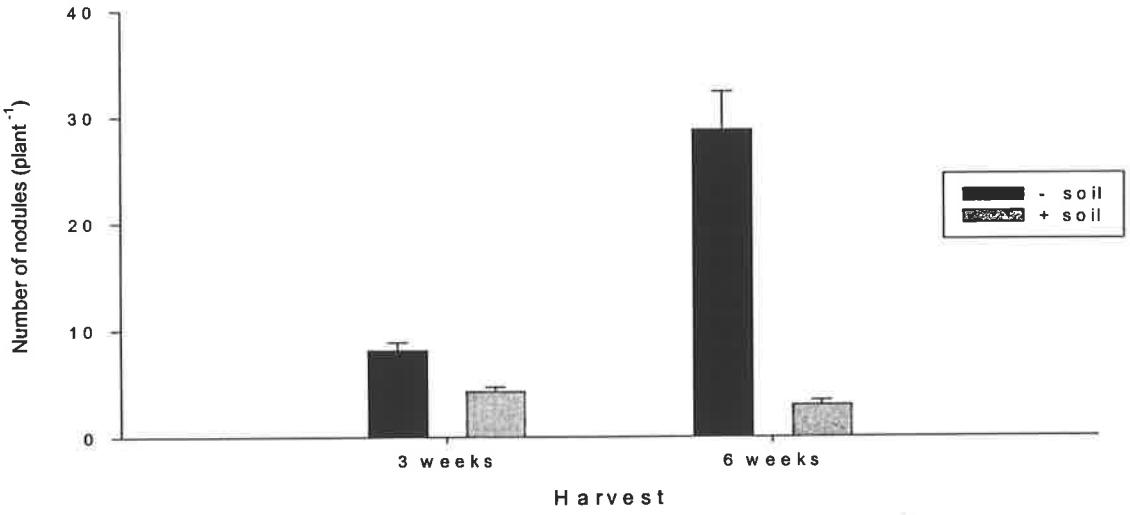


Figure 7.12: Presence of chlorsulfuron in the soil and harvest (3 and 6 weeks) interaction effects on nodule counts of chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

The number of nodules was expressed as a unit of root length and the mean results are presented in Table 7.6. As with the number of nodules per plant, pre-exposure of rhizobia to chlorsulfuron reduced the number of nodules per length of root, when the herbicide was absent from the soil and at germination (Table 7.6).

Table 7.6: Analysis of variance results for the effects of chlorsulfuron on the number of nodules per length of root.

Effect/interaction	p-value	Treatment		Mean number of nodules cm root^{-1}
Rhizobia*Soil	0.035	Rhizobia	soil	
		-	-	0.0362
		-	+	0.0206
		+	-	0.0276
		+	+	0.0303
Rhizobia*germination		Rhizobia	germination	
		-	-	0.0342
		-	+	0.0226
		+	-	0.0250
		+	+	0.0328

The fresh weight of nodules was affected by an interaction between rhizobia and nitrogen fertiliser (Table 7.5; Figure 7.13). Nitrogen fertiliser reduced the chickpea nodule weight of plants inoculated with non pre-exposed rhizobia. In the absence of nitrogen fertiliser, pre-exposing rhizobia reduced the nodule weight of chickpeas by 33%, but in the presence of nitrogen, pre-exposure of rhizobia increased nodule weight by 28% (Figure 7.13).

A significant interaction between rhizobia and germination also affected nodule weight (Table 7.5; Figure 7.14). When chlorsulfuron was absent at germination, pre-exposed rhizobia treatments had nodule fresh weights 33% lower than non pre-exposed rhizobia treatments. When chlorsulfuron was present at germination, pre-exposed rhizobia treatments had nodule weights that were 10% higher than non pre-exposed treatments (Figure 7.14). The nodule weight of plants grown in the presence of chlorsulfuron in the soil was 0.345 g plant^{-1}, and this represented a reduction of 68% compared to plants grown in the absence of chlorsulfuron in the soil, with an average nodule fresh weight of 1.060 g plant^{-1} (Table 7.5).

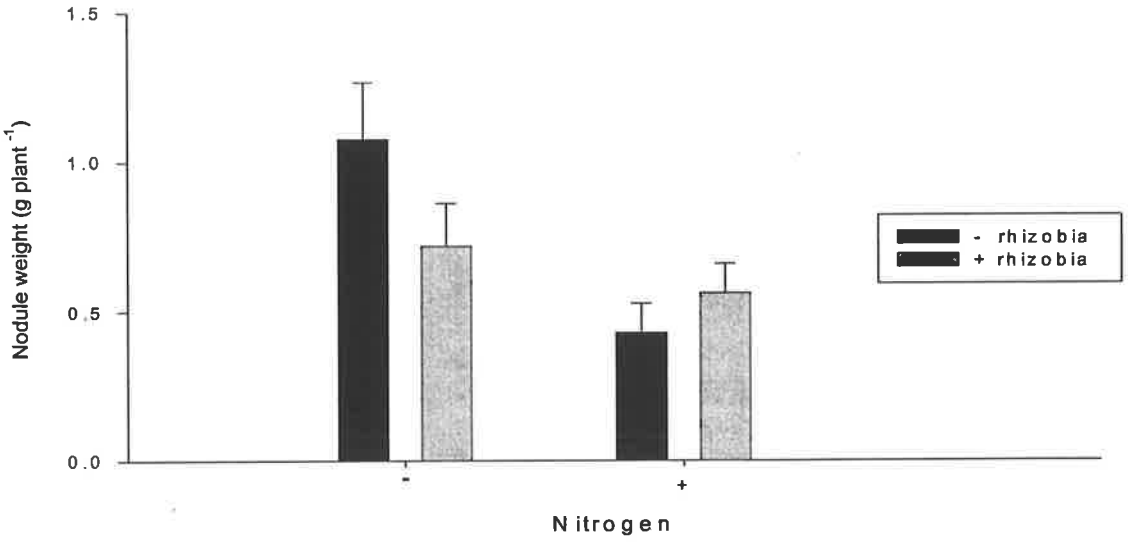


Figure 7.13: Interaction between rhizobia treatments (non pre-exposed (-), pre-exposed (+), uninoculated (0)) and nitrogen fertiliser (+/-) on nodule fresh weight of chickpea grown in a controlled environment room six weeks after inoculation. Bars indicate standard error of mean.

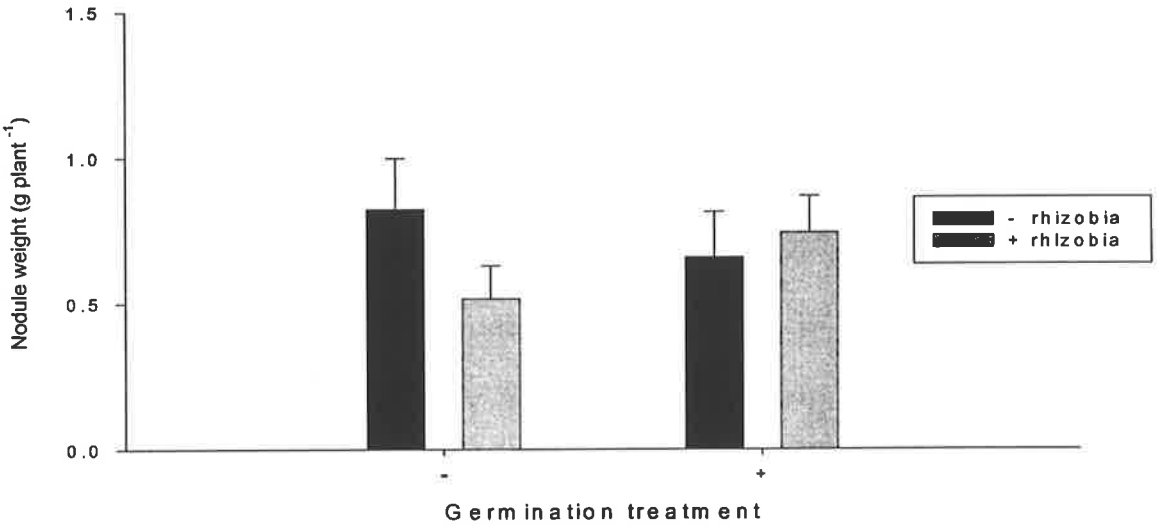


Figure 7.14: Effects of rhizobia (non pre-exposed (-), pre-exposed (+), uninoculated (0)) and germination(+/-) treatments on nodule fresh weight of chickpea plants six weeks after inoculation. Bars indicate standard error of mean.

Linear regression was used to determine if a relationship existed between nodule weight and nodule number and to investigate the changes in the slope and intercept of the lines to indicate if the relationship varies between the applied treatments. Reductions in nodulation and nitrogen fixation due to the presence of inorganic nitrogen in the soil have been well documented (Sprent and Sprent, 1990; Butler, 1988; Atkins, 1984; Jessop *et al.*, 1984). In fact, a nitrogen by rhizobia treatment interaction was noted in this study (Figure 7.15). As a result, the regression of nodule weight (g plant^{-1}) on the number of nodules (plant^{-1}) was completed separately for the data derived from the + and – nitrogen treatments. Significant positive linear relationships between nodule weight and number of nodules ($p<0.001$) were found in both the absence (Figure 7.17) and presence (Figure 7.18) of additional nitrogen, with r^2 values of 0.64 and 0.61 respectively.

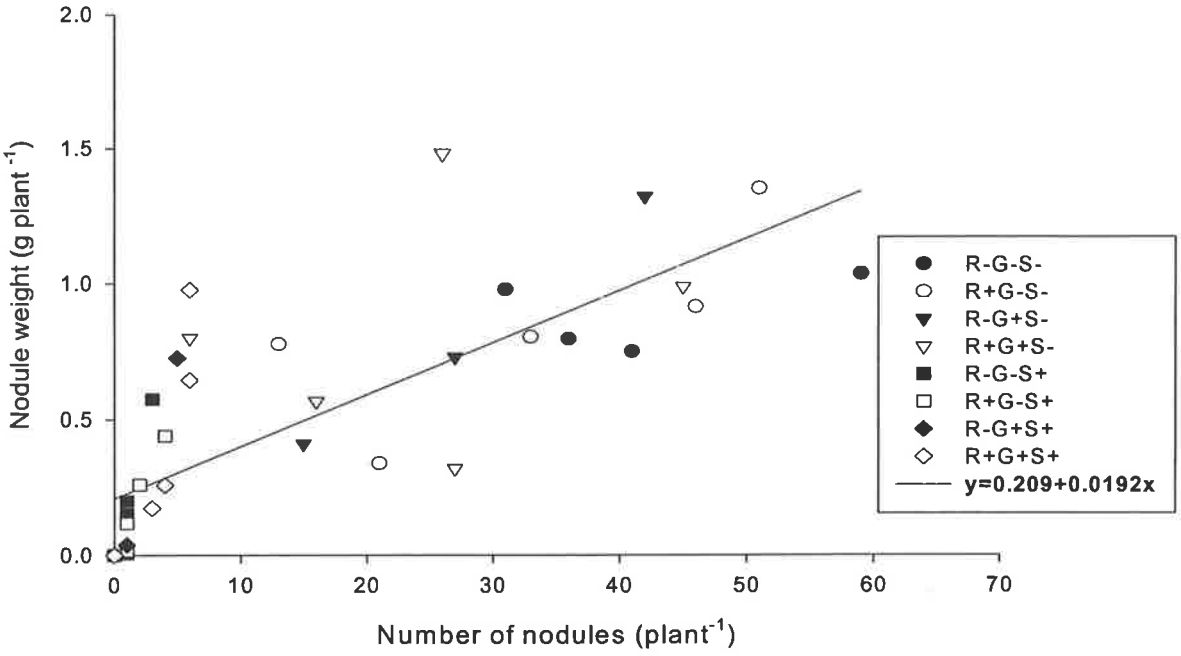


Figure 7.17: Relationship between total nodule weight and nodule number of chickpea plants grown in the absence of additional nitrogen fertiliser, 6 weeks after inoculation (r^2=0.64, p= <0.0001). R = rhizobia treatment; G = germination treatment; S= soil treatment. -/+ = absence and presence of chlorsulfuron . Standard errors of slope and intercept were 0.003 and 0.101 respectively.

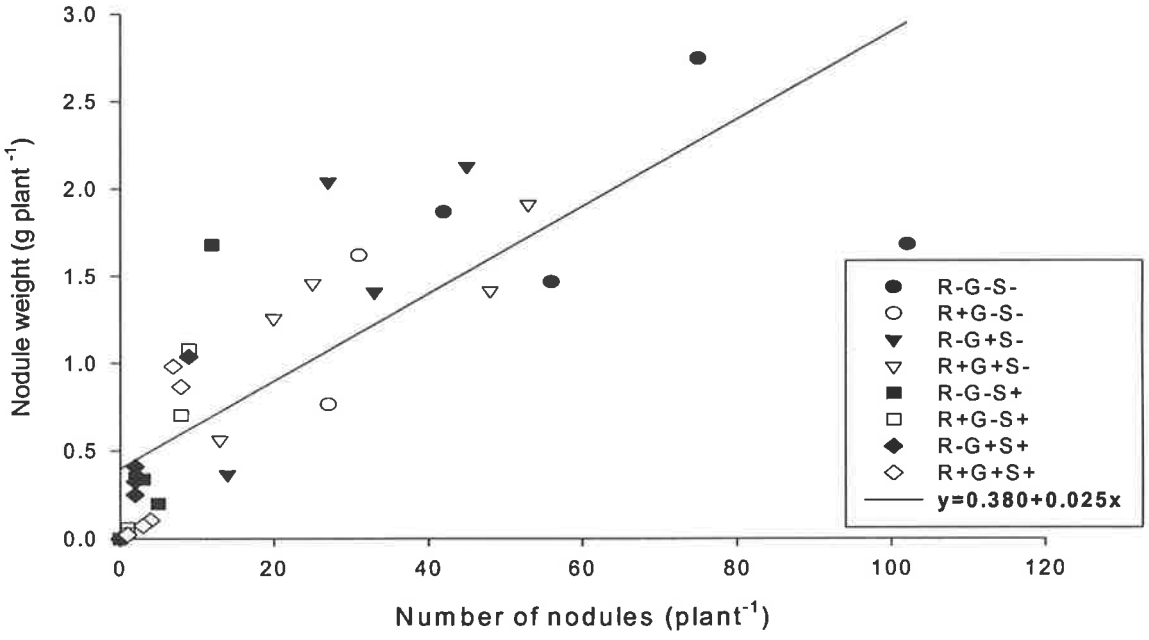


Figure 7.18: Relationship between nodule number and total nodule weight of chickpea plants grown in the presence of additional nitrogen fertiliser, six weeks after inoculation (r^2=0.61, p= <0.0001). R = rhizobia treatment; G = germination treatment; S= soil treatment. -/+ = absence and presence of chlorsulfuron. Standard errors of slope and intercept were 0.003 and 0.06 respectively.

7.3.4 Plant Nitrogen or Total Nitrogen.

Total plant nitrogen (combined roots and shoots) was significantly affected by four, three way interactions (Table 7.7). The presence of chlorsulfuron in the soil, nitrogen fertiliser and harvest interacted to affect plant nitrogen of chickpeas (Table 7.7; Figure 7.19). The presence of chlorsulfuron in the soil reduced plant nitrogen of chickpeas in comparison to those grown in the absence of chlorsulfuron, regardless of harvest time or the addition of nitrogen fertiliser (Figure 7.19). However, when the plants were grown without chlorsulfuron in the soil, plant nitrogen increased when nitrogen was present, and also with time, and was always greater than that found in plants grown in the presence of chlorsulfuron (Figure 7.19).

Table 7.7: Significant (α=0.05) effects of chlorsulfuron application on combined plant nitrogen of chickpea roots and shoots as determined by analysis of variance.

Variable	Source	P value
Total nitrogen (nitrogen content)	Soil*nitrogen*harvest	0.005
	Rhizobia*nitrogen*harvest	0.003
	Rhizobia*soil*harvest	0.021
	Rhizobia*germination*harvest	0.043
Estimate of nitrogen fixed	Soil	0.012

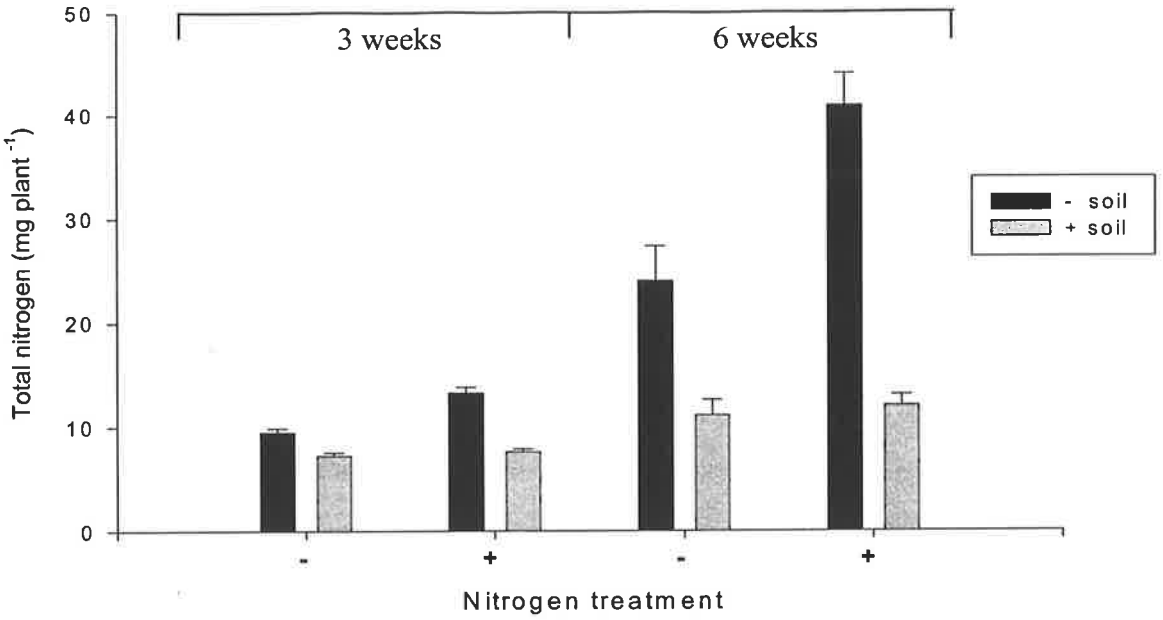


Figure 7.19: Effects of the presence and absence of chlorsulfuron in the soil, nitrogen fertiliser (presence (+)/absence (-)) and harvest time (3 and 6 weeks) on plant nitrogen of chickpea roots and shoots combined. Bars indicate standard error of mean.

Rhizobia treatments, nitrogen fertiliser and harvest time interacted to impact on plant nitrogen of chickpeas (Table 7.7). At the first harvest, the presence of nitrogen fertiliser resulted in only a small increase in chickpea plant nitrogen over all the rhizobia treatments (Figure 7.20). At the second harvest, plant nitrogen of chickpeas inoculated with non pre-exposed rhizobia treatments did not change with the addition of nitrogen fertiliser (Figure 7.20). However, the addition of nitrogen fertiliser resulted in a large increase in plant nitrogen in pre-exposed rhizobia (66%) and non inoculated treatments (150%) (Figure 7.20).

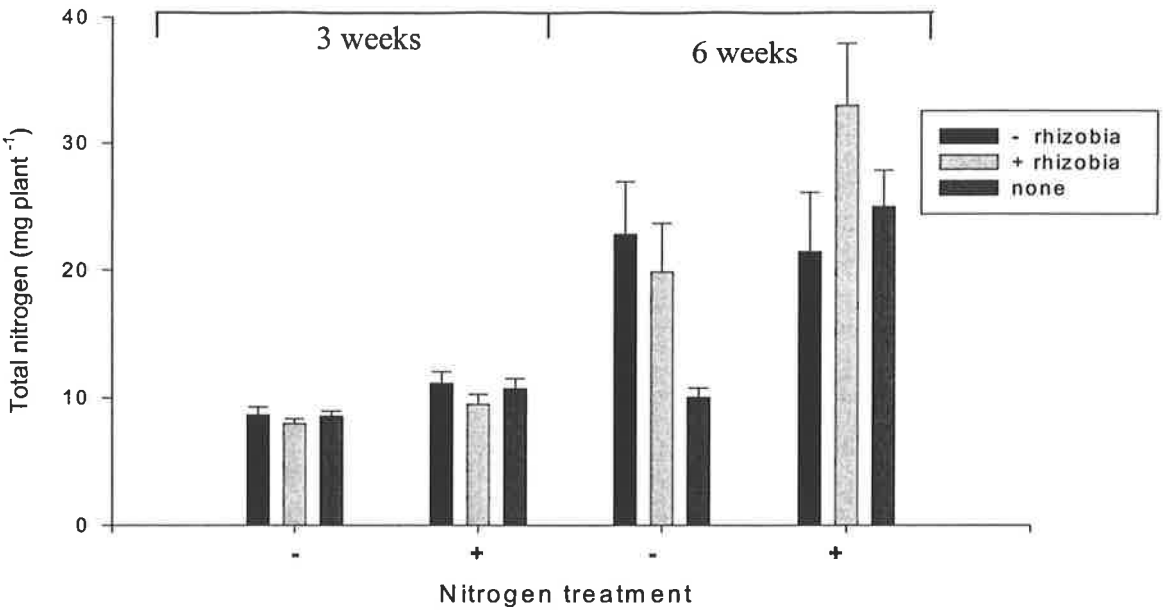


Figure 7.20: Effects of rhizobia (non pre-exposed (-), pre-exposed (+), uninoculated), addition of nitrogen fertiliser and harvest time (3 and 6 weeks) on plant nitrogen of chickpeas grown in a controlled environment room. Bars indicate standard error of mean.

Rhizobia treatments, presence of chlorsulfuron in the soil and harvest time interacted to affect plant nitrogen of chickpeas (Table 7.7; Figure 7.21). The presence of chlorsulfuron in the soil reduced the total plant nitrogen of chickpeas regardless of harvest time or rhizobia treatment (i.e. pre-exposed, non pre-exposed and non inoculated treatment), with the difference magnified at the second harvest (Figure 7.21). At the second harvest, and in the absence of chlorsulfuron from the soil, higher plant nitrogen was found in plants inoculated with pre-exposed rhizobia treatments than in the non-pre-exposed (17% lower) and non inoculated (40% lower) treatments (Figure 7.21).

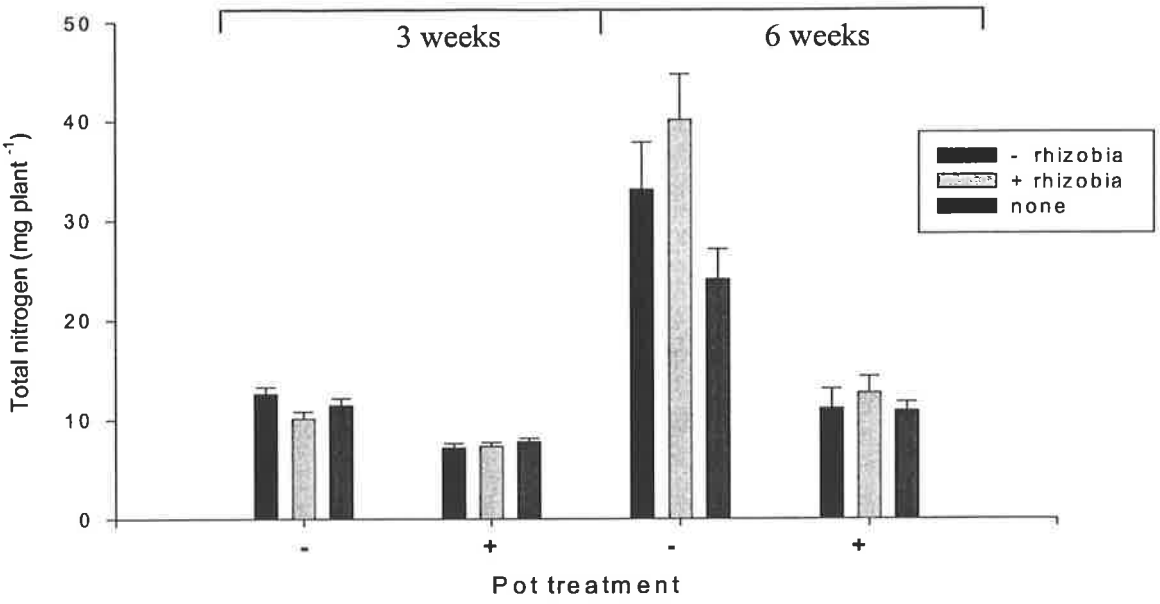


Figure 7.21: Effects of rhizobia (non pre-exposed (-), pre-exposed (+), uninoculated), presence and absence of chlorsulfuron in the soil and harvest (3 and 6 weeks) interact to affect plant nitrogen of chickpeas. Bars indicate standard error of mean.

Rhizobia, germination treatments and harvest time interacted to affect the plant nitrogen of chickpeas (Table 7.7; Figure 7.22). At the time of the first harvest, there was little difference between rhizobia or germination treatments in the presence or absence of chlorsulfuron (Figure 7.22). However at the second harvest, when chlorsulfuron was absent at germination, the plants inoculated with non-pre-exposed rhizobia had the highest plant nitrogen. When chlorsulfuron was present at germination however, the plant nitrogen of chickpeas was highest in plants inoculated with pre-exposed rhizobia (Figure 7.22).

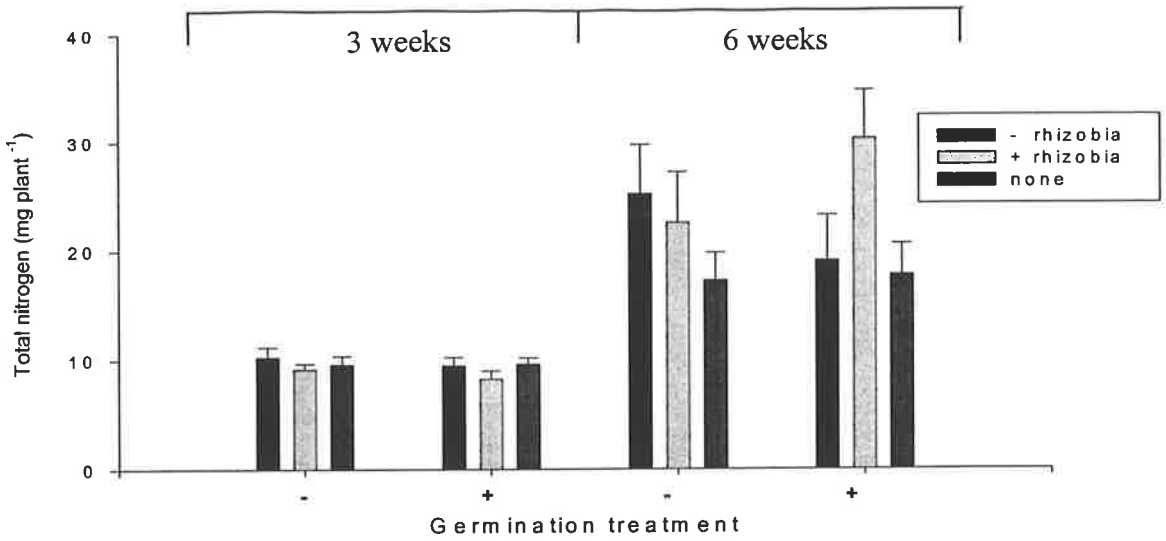


Figure 7.22: The effects of rhizobia and germination treatments and harvest time on plant nitrogen of chickpeas. The rhizobia treatments were: non pre-exposed (-), pre-exposed (+) and uninoculated. The germination treatments were: germinated in the presence (+) and absence (-) of chlorsulfuron. The harvest times were at 3 and 6 weeks. Bars indicate standard error of mean.

The % N data from harvest 2 (Table 7.8) show similar trends to that of the total plant nitrogen at the second harvest described above. Rhizobia and nitrogen treatments interacted to affect the %N of chickpea plants. In the absence of nitrogen fertiliser, the mean % N is lowest in uninoculated treatments (up to 50% reduction in comparison to treatments inoculated with rhizobia not pre-exposed to chlorsulfuron), whilst in the presence of nitrogen fertiliser the reduction of %N in uninoculated plants was only 10% when compared to the plants inoculated with non pre-exposed rhizobia (Table 7.8). Rhizobia and germination treatment also interacted to affect % N of chickpea plants (Table 7.8). In the absence of chlorsulfuron at germination, the uninoculated plants had % N values 35% lower than that of plants inoculated with non pre-exposed rhizobia (Table 7.9). However, when herbicide was present at germination, the plants inoculated with rhizobia pre-exposed to chlorsulfuron had the highest % N with values 23% higher than those not pre-exposed to chlorsulfuron (Table 7.8). The presence of chlorsulfuron in the soil reduced % N of chickpea plants by 16% (Table 7.8).

Table 7.8: Significant effects of chlorsulfuron on % N of chickpeas 6 weeks after inoculation

Effect/Interaction	p-value	Treatment		Mean
Rhizobia*Nitrogen	**0.001**	**Rhizo**	**N**	**(% N)**
		-	-	4.93
		+	-	4.17
		none	-	2.39
		-	+	5.26
		+	+	6.09
		none	+	4.71
Rhizobia*Germination	**0.034**	**Rhizo**	**Germ**	**Mean**
		-	-	5.55
		+	-	4.54
		none	-	3.62
		-	+	4.64
		+	+	5.72
		none	+	3.47
Soil	**0.004**	**Soil**		**Mean**
		-		5.00
		+		4.18

There was a significant main effect of the presence of chlorsulfuron in the soil on the estimate of nitrogen fixed by chickpea plants (Table 7.7). The estimate of nitrogen fixed when chlorsulfuron was present in the soil, was 6.04 mg plant^{-1}, which represents a 70% reduction compared to plants grown without chlorsulfuron in the soil (19.73 mg plant^{-1}).

7.3.5 Plant nitrogen and nodule weight

To give an indication of the effects of the chlorsulfuron on the efficiency of the nodules (nodule formation and indirectly nitrogen fixation), linear regression analyses were performed to determine if there was a relationship between plant nitrogen and nodule weight six weeks after inoculation and to allow comparison of the slopes and intercepts of different treatments. The data were initially split on the basis of nitrogen (Figures 7.23 and 7.24; Table 7.9), due to nitrogen interactions with other treatments affecting plant nitrogen of chickpeas (Table 7.9). The data were then split on the basis

of soil treatment and rhizobia treatment also due to interaction effects on plant nitrogen (Table 7.9). Significant positive linear relationships of plant nitrogen on nodule weight (g plant^{-1}) were obtained for both absence (Figure 7.23; Table 7.9) and presence of additional nitrogen (Figure 7.24; Table 7.9); rhizobia not pre-exposed and pre-exposed (Table 7.9); and both presence and absence of chlorsulfuron in the soil (Table 7.9), demonstrating that plant nitrogen was positively related to nodule weight.

Table 7.9: Regression table for nodule weight against plant nitrogen for chickpea plants. (i) separated on the basis of nitrogen treatment with all other treatments included; (ii) separated on the basis of rhizobia treatment (pre-exposed and non pre-exposed to chlorsulfuron; (iii) separated on the basis of soil treatment (Presence (+) and absence (-) of chlorsulfuron in the soil. (α=0.05). Numbers in parentheses indicate standard error.

Treatment		Slope	Intercept	r^2	P value
No additional nitrogen	(i) All treatments (i.e. +/- rhizobia and soil)	20.335 (1.254)	-4.081 (1.463)	0.89	<0.0001
	(ii) - Rhizobia	19.007 (1.577)	-4.158 (2.142)	0.91	<0.0001
	+ Rhizobia	23.852 (1.911)	-5.187 (1.819)	0.91	<0.0001
	(iii) - Soil	18.60 4 (2.100)	0.226 (3.210)	0.84	<0.0001
	+ Soil	16.973 (1.811)	-4.373 (1.201)	0.85	<0.0001
Additional nitrogen	(i) All treatments (i.e. +/- rhizobia and soil)	43.486 (3.826)	-3.586 (2.528)	0.78	<0.0001
	(ii) - Rhizobia	43.731 (4.268)	-5.455 (2.583)	0.87	<0.0001
	+ Rhizobia	42.13 0 (6.363)	-1.237 (4.500)	0.71	<0.0001
	(iii) - Soil	37.753 (4.940)	6.531 (4.230)	0.77	<0.0001
	+Soil	18.530 (2.371)	-3.352 (0.888)	0.78	<0.0001

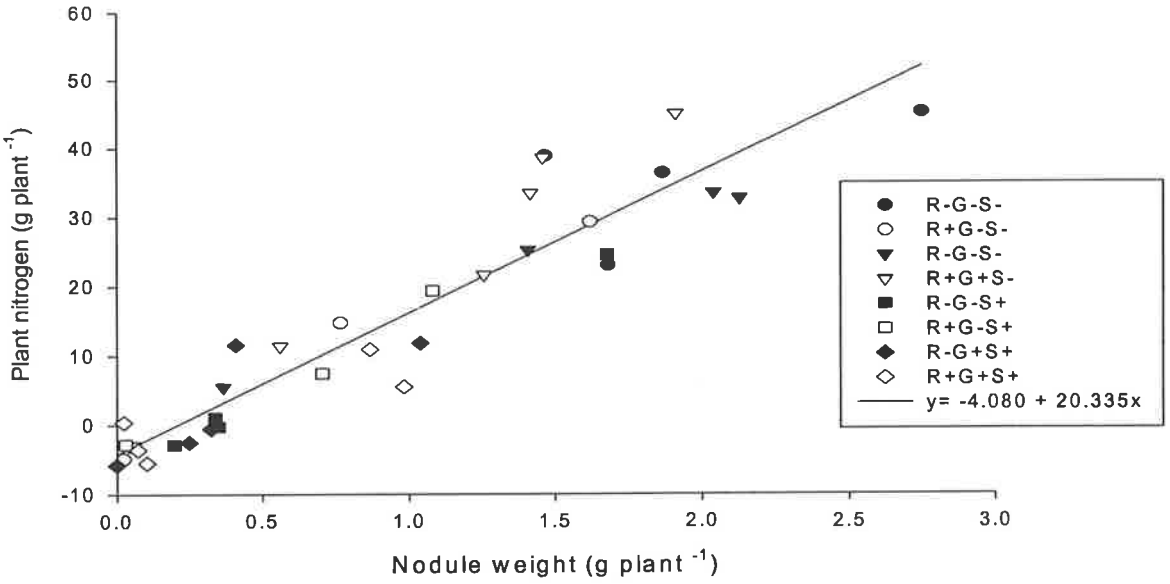


Figure 7.23: Relationship between nodule weight and plant nitrogen of chickpeas grown in the absence of additional nitrogen fertiliser, six weeks after rhizobial inoculation. (r^2 = 0.8885; p <0.0001). R = rhizobial treatment; G= germination treatment; S = soil treatment; +/- = presence and absence of chlorsulfuron respectively.

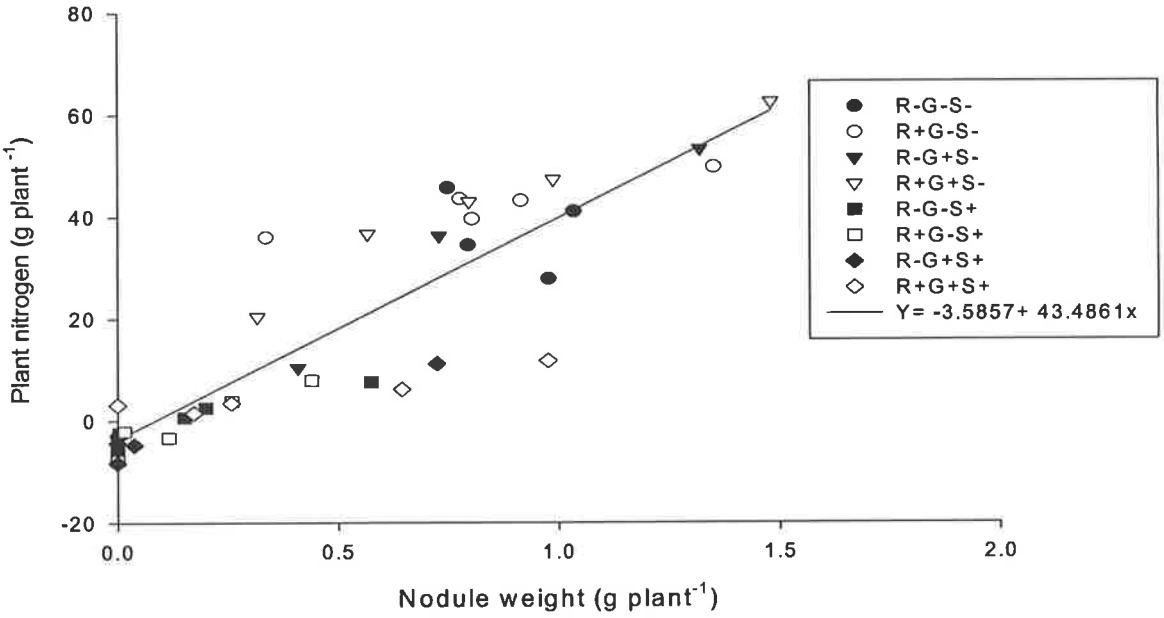


Figure 7.24: Relationship between nodule weight and plant nitrogen of chickpeas grown in the presence of additional nitrogen fertiliser, six weeks after rhizobial inoculation. (r^2 = 0.7821; p <0.0001). R = rhizobial treatment; G= germination treatment; S = soil treatment; +/- = presence and absence of chlorsulfuron respectively.

7.4 DISCUSSION

7.4.1 Effects of chlorsulfuron on plant biomass, shoot area and root length density

Sulfonylureas are absorbed by both the roots and shoots of plants and are readily translocated via the xylem and phloem (Devine *et al.*, 1993; Brown, 1990; Ray, 1982a). The mode of action of sulfonylurea herbicides is through the inhibition of acetolactate synthase, an enzyme responsible for the biosynthesis of the branched chain amino acids valine, leucine and isoleucine (Devine *et al.*, 1993; Mousdale and Coggins, 1991; Brown, 1990; LaRossa and Schloss, 1984; Ray, 1984; Ray, 1982). In green plants, branched-chain amino acids are synthesised in the chloroplasts (Mousdale and Coggins, 1991). The inhibition of acetolactate synthase, therefore, results in a rapid cessation in cell division and growth (Brown, 1990; Beyer *et al.*, 1988). Rost (1984) hypothesised that chlorsulfuron inhibits cell cycle progression by inhibiting the G_2 (pre-mitosis phase when cells are metabolically preparing for mitosis (Weier *et al.*, 1982)) and G_1 (pre-DNA synthesis phase when the cell is metabolically preparing itself for DNA synthesis (Weier *et al.*, 1982)) transition points through inhibition of cell cycle specific RNA progression. Kim and Vanden Born (1997 a and b) reported that chlorsulfuron reduced assimilate transport from treated canola leaves and that the reduction in growth of chlorsulfuron treated canola can be attributed, at least in part, to carbohydrate starvation of rapidly growing tissue in sink areas.

Chlorsulfuron affected the growth and nodulation of chickpea plants in the study discussed in this chapter. Although the experiment was only undertaken for a period of six weeks, effects of herbicide were visible at the second harvest and the effects would probably have become more obvious had the experiment continued. The presence of

chlorsulfuron in the soil, at levels equivalent to residues one year after application, reduced plant biomass, shoot area and root length density of chickpea plants. The biomass reduction due to chlorsulfuron increased over time and the addition of nitrogen fertiliser failed to alleviate herbicide effects. The root length density and root biomass of chickpeas were inhibited by chlorsulfuron in the soil to a greater extent than shoot biomass. The roots came into direct contact with the soil applied herbicide and would therefore exhibit symptoms of herbicide effects earlier than the shoots. The reduction in root biomass and root length density, due to herbicide effects on cell division, decreased the ability of the plants to absorb nutrients, and would explain why the addition of nitrogen fertiliser did not increase plant biomass or shoot area, following chlorsulfuron inhibition. The reduced plant nitrogen of the chickpeas grown in the presence of chlorsulfuron, even when nitrogen fertiliser was added, demonstrates the decreased ability of root systems to absorb nutrients.

Pre-exposing rhizobia to chlorsulfuron, in this study, also reduced root biomass of chickpea plants in comparison to plants that were inoculated with non pre-exposed rhizobia. The plants inoculated with pre-exposed rhizobia also responded more to nitrogen fertiliser through an increase in root and shoot biomass and this may indicate ineffective symbiosis and nitrogen fixation. Plants that were not inoculated had lower root and shoot biomass than those that were inoculated. This lower biomass was probably due to a lack of symbiotic nitrogen fixation in uninoculated plants and, therefore, the plant relied on nitrogen from seed reserves or the soil. Root length density of chickpea plants not inoculated and those inoculated with pre-exposed rhizobia responded more to the addition of nitrogen fertiliser than those that were

inoculated with pre-exposed rhizobia. In the absence of nitrogen, pre-exposing rhizobia to chlorsulfuron reduced nodulation.

Reduction in chickpea root and shoot biomass from the application of chlorsulfuron is supported by results from other studies of ALS-inhibiting herbicides. Chlorsulfuron at approximately 1% of the recommended rate of application to cereals was found to reduce root weight and length of the tap root of *Medicago truncatula* plants by more than 50% compared to control treatments (Rovira *et al.*, 1993). Gillett and Holloway (1996) found triasulfuron at 1% and 4% of the recommended rate (incorporated in 10 – 20 cm layer of soil in cylindrical pots) significantly reduced shoot dry matter by 18% and 26% respectively and restricted root penetration of medic. In another study, triasulfuron (4 g a.i. ha^{-1}) and chlorsulfuron (11 g a.i. ha^{-1}) reduced annual medic shoot dry matter by 58% and 78% respectively, at pH 8.5 – 9.5, twelve months after application to a wheat crop (Evans *et al.*, 1993).

When plants were not inoculated or inoculated with pre-exposed rhizobia, in the study discussed in this chapter, the addition of nitrogen fertiliser increased shoot and root biomass, shoot area and root density of chickpea. These increases suggest that effective nitrogen fixing symbioses were not formed and plant response to the additional nitrogen supports this finding. The shoot biomass, shoot area, root biomass and root density of chickpea plants, inoculated with non pre-exposed rhizobia, did not increase with the addition of nitrogen fertiliser, suggesting that the plants could meet their nitrogen demand with symbiotic nitrogen fixation. This is also supported by the data which indicated that total N and % N also did not increase indicating these plants are not taking up additional available N.

The presence of chlorsulfuron in the soil, in which the chickpea plants were grown, severely reduced the shoot area of chickpea plants. The addition of nitrogen did not alleviate this reduction. The addition of nitrogen fertiliser increased shoot area of chickpea plants in the absence of chlorsulfuron. Mckenzie *et al.* (1992), found that an increased leaf area index of Kabuli type chickpea resulted in an increase in intercepted solar radiation, which in turn increased dry matter production. In the current study, residual levels of chlorsulfuron were found to inhibit shoot area and also shoot and root biomass. Chlorsulfuron is transported to shoots from the roots and would directly affect the growth of the meristem (Devine *et al.*, 1993; Brown, 1990). The reduction in root biomass was probably most critical, in that, it prevented the plant from taking up nutrients from the soil and in turn reduced shoot biomass production. This is supported by evidence that chlorsulfuron has been found to selectively inhibit the cell cycle in root tips (Rost *et al.*, 1990) without apparently affecting any other metabolic process such as stem elongation and leaf expansion (Rost *et al.*, 1984; Ray, 1982a). As root biomass was more severely reduced than shoot biomass and the roots were the first to come into contact with chlorsulfuron, it is likely that the reduction in growth would start with the roots and eventually spread to the shoots. When assimilates are partitioned in favour of leaf area development, the rate of dry matter accumulation increases exponentially over time as the increased leaf area assimilates more carbon (Khanna Chopra and Lakshmi, 1987). Thomas and Fukai (1995), found that the leaf area of chickpea plants was reduced by water stress, which in turn reduced interception of photosynthetically active radiation and hence reduced dry matter production. It is possible that in the study presented in this chapter, the reduction in leaf area by chlorsulfuron, led to the reduction in shoot biomass.

7.4.2 Chlorsulfuron effects on nodulation and nitrogen

Chlorsulfuron reduced the number of nodules found on chickpea roots. The greatest reduction was found when the herbicide was present in the soil. However, the number of nodules per root length increased in the presence of chlorsulfuron in the soil, and this indicates that the reduction in nodulation may be due to a reduction in available infection sites. There are difficulties with interpreting this however, and a more appropriate comparison may have looked at the number of root branches or root hairs, rather than root length.

Additional nitrogen also inhibited nodulation. Nodulation of chickpeas is known to decrease with increasing levels of nitrogen (Jessop *et al.*, 1984). Results from other authors support the reduction in nodulation from chlorsulfuron. Eberbach and Douglas (1989), found that nodulation of *Trifolium subterraneum* was reduced by chlorsulfuron at concentrations of 0.2 – 2.0 mg ai L^{-1}, where 0.2 mg ai L^{-1} is equivalent to concentrations in the top 1cm of soil following recommended field applications. These concentrations are higher than those used in this pot trial at 10% of the recommended application rate for chlorsulfuron.

Chlorsulfuron at application rates of 1 x 10^{-7} and 1 x 10^{-9} mg L^{-1} has been shown to reduce the bacterial root hair deformations of red clover (*Trifolium pratense*) grown in growth chambers prepared using microscope slides by the Fahraeus method (Martensson, 1992). Martensson and Nilsson (1989), found that levels of chlorsulfuron of 500 and 5000 times the recommended field application rates resulted in turgid root tips and thick roots with few, abnormally curved lateral roots in red clover and lucerne (*M. sativa*) plants. These levels were unrealistically high compared to recommended field application rates. The same authors found that chlorsulfuron rates of 2 x 10^{-6} and 2

x 10^{-3} g ha^{-1} did not affect the number of nodules of lucerne plants, but rates of 2 g ha^{-1} reduced the number of nodules by 77% compared to a control treatment and rates of 4 and 8 g ha^{-1} resulted in no nodules forming at all (Martensson and Nilsson, 1989). In the study discussed in this chapter, nodulation was reduced by chlorsulfuron applied to the soil at an application rate of 1.5 g ai ha^{-1} and when rhizobia were pre-exposed to chlorsulfuron at a rate of 15 g ai ha^{-1}.

Reduction in chickpea nodulation (in the study discussed in this chapter) was observed when rhizobia were pre-exposed to chlorsulfuron *in-vitro*. When chlorsulfuron was not present at any other stage (i.e. in the soil or during germination), the reduction in number of nodules and nodules per root length were still observed when chickpea plants were inoculated with rhizobia pre-exposed to chlorsulfuron. The reduction in nodules per root length indicates that the reduced nodulation was not due to reduced infection sites and may alternatively be due to reduced ability to initiate nodules. The pre-exposed rhizobia were rinsed with a saline (Ringer's) solution three times before inoculation to remove any residual herbicide. The reduction, due to the pre-exposure of rhizobia to chlorsulfuron, could be due to a number of factors, including a direct effect of chlorsulfuron on the growth or survival of rhizobia, carryover of chlorsulfuron on the rhizobial cells even after rinsing and therefore to the point of infection, or thirdly by chlorsulfuron influencing the nodule formation process. These factors will be discussed in more detail in later chapters.

The fresh weight of nodules was also reduced by the pre-exposure of rhizobia to chlorsulfuron, as well as from the presence of chlorsulfuron in the soil. This reduction in fresh weight is probably related directly to the observed reduction in numbers of nodules. Chlorsulfuron also reduced plant nitrogen of inoculated chickpeas grown in

the absence of nitrogen, which could imply that the herbicide reduced the amount of nitrogen fixed by the plant.

The relationship between number and weight of nodules of chickpeas showed that the slopes of both the + and – nitrogen treatments were similar, suggesting that nitrogen did not affect the relationship between weight and number (i.e. for a given number of nodules the weight will be the same with or without additional nitrogen). In the absence of nitrogen, the plants inoculated with non pre-exposed rhizobia had both high weights and numbers of nodules. However, the chickpea plants inoculated with pre-exposed rhizobia generally had both lower nodule numbers and weights. These results show that the pre-exposure of rhizobia to chlorsulfuron affects the nodulation of chickpeas. The distinct difference between pre-exposed and non pre-exposed rhizobia treatments did not exist in the presence of nitrogen. This may be due to the inhibitory effects of inorganic nitrogen (Sprent and Sprent, 1990; Atkins, 1984; Jessop *et al.*, 1984) being greater than the effects of growing rhizobia in the presence of chlorsulfuron.

When chlorsulfuron was present in the soil (with and without additional nitrogen), both number and weight of nodules were low. Some plants had a few large nodules, thus leading them to lie above the regression line. In these cases, it is possible that in a response to herbicide effects, the plant is putting more energy into creating larger nodules than forming new ones. The potential for a nodule to become large may serve as an insurance against adverse conditions where the number of nodules formed is limited (Rupela and Saxena, 1987). The larger nodules found on chickpea plants grown in the presence of chlorsulfuron in the soil, were often black in colour. The upper roots

of the chickpea plants grown in soil to which chlorsulfuron was applied were also black and this may be a symptom of herbicide application.

In many legumes, nodule mass is better correlated with biological nitrogen fixation than nodule number per plant (Pate, 1977). In this study, the slope of the regression of plant nitrogen on nodule weight of chickpea plants grown with nitrogen fertiliser was twice that of the plants grown without nitrogen fertiliser. The nitrogen fertiliser, therefore, provided a source of nitrogen that the plants used in addition to symbiotically fixed nitrogen.

The regression performed on plants grown in the absence of nitrogen, showed that plants inoculated with non pre-exposed rhizobia had both high nodule numbers and high plant nitrogen. The plants inoculated with pre-exposed rhizobia had low nodule numbers and low plant nitrogen. However, when taking into account the standard errors of the slopes of the graphs, the pre-exposed rhizobia treatment slope was higher than that of the non pre-exposed treatment. This higher slope indicates that each unit of nodule weight in the pre-exposed treatment was capable of fixing more nitrogen, compared to that of the non pre-exposed rhizobia treatment. So, although number and weight of nodules were reduced, the nodules may have been compensating by fixing more nitrogen per unit of nodule weight. The intercepts for the relationship of plant nitrogen on nodule weight, were negative for both the pre-exposed and non-pre-exposed rhizobia treatments, indicating that without nodules, nitrogen was not fixed.

The regression of plant nitrogen on nodule weight for chickpea plants grown in soil with chlorsulfuron (and in the absence of additional nitrogen), showed that plants had low nodule weights and low plant nitrogen, with most falling below the regression

line. The plants grown without chlorsulfuron in the soil had high nodule weights and high plant nitrogen. The slopes of the two regressions (+/- chlorsulfuron in the soil) were similar, suggesting that in the absence of nitrogen each unit of nodule weight is capable of fixing the same amount of nitrogen. However, the low nodule weight still led to less plant nitrogen overall compared to those plants grown without chlorsulfuron in the soil.

However, in the presence of nitrogen, the slope of the regression (plant nitrogen on nodule weight) of plants grown with chlorsulfuron in the soil, was approximately half that of those grown without chlorsulfuron in the soil. This suggests that the nodules of plants grown with chlorsulfuron in the soil, were capable of fixing only half the amount of nitrogen as those grown without chlorsulfuron in the soil. Alternatively, chlorsulfuron reduced plant root biomass and the roots were incapable of taking up the available nitrogen and, therefore, the untreated plants had a higher slope. The negative intercept of the regression (plant nitrogen on nodule weight) with chlorsulfuron in the soil, as opposed to the positive intercept without chlorsulfuron, suggests that in the presence of chlorsulfuron the plant is not taking up available nitrogen from the soil, probably due to reduced root biomass.

The slopes of the regressions (plant nitrogen on nodule weight) for pre-exposed and non pre-exposed rhizobia treatments were similar in the presence of nitrogen. Thus, in the presence of nitrogen, pre-exposure of rhizobia to chlorsulfuron did not affect the amount of nitrogen fixed or taken up per unit weight of nodule.

The reduction in number and mass of nodules and amount of nitrogen in chickpea plants has consequences for the growth and grain yield of chickpeas as well as

the proportion and amount of nitrogen fixed. This may lead to reduced amounts of inorganic nitrogen being available for future crops or reduced amounts of fixed nitrogen. Chickpea shoots have an average of 35 k N ha^{-1} of soil derived nitrogen (Total N – fixed N in peak aboveground biomass) in New South Wales (Armstrong *et al.*, 1997). Armstrong *et al.* (1997), also noted that the residual N balance of the chickpea crops through the return of non-harvested aboveground biomass (fixed N in peak aboveground biomass minus N removed as grain) was up to 33 kg ha^{-1} (range of –12 – 33 kg ha^{-1}) with the lower values coming from a later sowing. Schwenke *et al.*(1998), have reported net N balances for chickpea in NSW with a range of –47 to 46 kg N ha^{-1}. If the use of chlorsulfuron reduces the number and weight of nodules and therefore, nitrogen fixation of chickpeas, a reduction in any nitrogen benefits from the crop will be observed.

7.5 SUMMARY AND KEY FINDINGS

Pre-exposing rhizobia to chlorsulfuron reduced the number of nodules formed on chickpea roots in the absence of chlorsulfuron in the soil or at germination. This result has not previously been investigated or observed and may be unique to chickpeas. The reduction in the number of nodules due to pre-exposure of rhizobia may be due to a number of factors, which will be discussed in the following chapters. This pre-exposure of rhizobia also led to reduced % N of chickpea plants, indicating that plant nitrogen relations may be affected. Inoculation of chickpeas is considered essential the first time the crop is grown in a paddock and as an insurance the next time the crop is sown (Lamb and Poddar, 1992). However, inoculation after the second time is only recommended if conditions adverse to natural rhizobia exist (Lamb and Poddar, 1992). When chlorsulfuron is applied to cereal crops, natural populations of rhizobia will be exposed to the herbicide and future nodulation of chickpea plants may be inhibited.

The presence of chlorsulfuron in the soil reduced shoot and root biomass and the number of nodules of chickpea plants. The reduction in nodulation from the presence of chlorsulfuron in the soil, is likely a response to the reduction in plant biomass leading to reduced root infection sites. For a legume to effectively fix nitrogen, it needs to be well nodulated, have sufficient photosynthate, respire efficiently, be exposed to optimal environmental conditions (temperature, water supply etc.), and have an efficient vascular system for transport of products into and out of nodules and redistribution throughout the plant (Sprent and Sprent, 1990). The results from this study have shown that chlorsulfuron reduced nodulation, root biomass, shoot area and shoot biomass. In addition, other factors required for effective nitrogen fixation may be adversely

affected. The use of chlorsulfuron may have consequences for chickpea growth, grain yield, nitrogen fixation and the future nitrogen balance of the soil.

Key Findings:

- The pre-exposure of rhizobia to chlorsulfuron reduced the number of nodules formed on chickpea plants, in the absence of herbicide in the soil or at germination.
- The presence of chlorsulfuron in soil reduced shoot and root biomass, shoot area and root length density.
- The number of nodules was reduced by the presence of chlorsulfuron in the soil
- Plant nitrogen of chickpeas was reduced by the presence of chlorsulfuron in the soil.
- The estimate of nitrogen fixed was reduced by the presence of chlorsulfuron in soil.

The next chapter will investigate the effects of imazethapyr on the ability of rhizobia to nodulate chickpeas and Chapter 9 will investigate the factors which may be responsible for the reduction in nodulation due to pre-exposure of rhizobia.

CHAPTER 8

THE EFFECTS OF IMAZETHAPYR ON CHICKPEA GROWTH, NODULATION AND *RHIZOBIUM*

8.1 INTRODUCTION

It has been previously demonstrated that the ALS-inhibiting herbicide imazethapyr reduced the growth of chickpea and inhibited nitrogen fixation in the field (Chapter 5). Imazethapyr, unlike the sulfonylurea herbicide chlorsulfuron, belongs to the imidazolinone family of herbicides (Hart *et al.*, 1991; Rost *et al.*, 1990). The mode of action of inhibition of acetolactate synthase is the same as the sulfonylureas (Stidham and Singh, 1991; Rost *et al.*, 1990; Stidham and Shaner, 1990). It is believed that the imidazolinones kill plants by being retained in the phloem until the compounds reach the meristem (Little and Shaner, 1991).

Few studies have investigated the effects of imidazolinone herbicides on the process of nodule formation. The nodule numbers of *M. truncatula* cultivars Caliph, Mogul and Paraggio were reduced by imazethapyr (Fajri *et al.*, 1996). Gonzalez *et al.* (1996), found that imazethapyr affected the number of nodules on pea (*Pisum sativum*) plants more than the size of nodules and suggested this was the result of the herbicide affecting nodule initiation rather than nodule development. The results of these studies suggested that imazethapyr may inhibit nodule formation in the legumes studied, even though rhizobial populations were not necessarily affected.

The objectives of the experiment in this chapter were to quantify whether imazethapyr affected growth and nodulation of chickpea in a manner similar to that observed for chlorsulfuron in Chapter 7.

8.2 MATERIALS AND METHODS

The experimental design, materials and methods used in this experiment were identical to those used for the chlorsulfuron experiment discussed in Chapter 7 (section 7.2). The only difference between the two experiments was the application rate of the herbicides. Table 8.1 summarises the imazethapyr application rates and the sampling protocol for the experiment is presented in Table 8.2.

See Chapter 7 for details on treatments, experimental design, measurements and data interpretation.

Table 8.1: Imazethapyr application rates at each stage of herbicide application (rec. app. rate = recommended application rate).

Stage	Imazethapyr	
Rhizobia	0.02 µg ai ml^{-1}	Rec. app. rate
Germination	0.02 µg ai ml^{-1}	Rec app rate
Soil/pot	0.02 µg ai ml^{-1}	Rec app rate

Table 8.2: Summary of samples taken at three and six week harvests for imazethapyr experiment (4 indicates that samples were taken).

Parameter	3 weeks	6 weeks
Shoot biomass	4	4
Shoot area	4	4
Root biomass	4	4
Root length	4	4
Nodule count	4	4
Nodule fresh weight		4
Total nitrogen		4
Nitrogen fixed (inference)		4

8.3 RESULTS

8.3.1 Shoot biomass and total shoot area

There were no significant interactions or main effects of imazethapyr application on shoot biomass of chickpeas. Total shoot area was affected by a three-way interaction between rhizobia, germination treatments and harvest time (Table 8.3; Figure 8.1). At the first harvest, the total shoot area of chickpeas inoculated with non pre-exposed rhizobia treatments increased when imazethapyr was present at germination compared to those that were germinated without imazethapyr (Figure 8.1). At the first harvest, the total shoot area of chickpea plants inoculated with rhizobia pre-exposed to imazethapyr, decreased when imazethapyr was present at germination. The shoot area of non-inoculated treatments remained similar in both the presence and absence of imazethapyr at germination (Figure 8.1). At the second harvest non pre-exposed and non-inoculated rhizobia treatments had similar shoot area, when imazethapyr was both present and absent at germination (Figure 8.1). The pre-exposed rhizobia treatments increased in shoot area when imazethapyr was present at germination when compared to absence at germination (Figure 8.1).

Table 8.3: Significant (α=0.05) effects of imazethapyr application on total shoot area of chickpea plants as determined by analysis of variance.

Variable	Source	P value
Shoot area	Rhizobia*germination*harvest	0.006
	Nitrogen*harvest	0.007
	Soil*harvest	<0.001
	Soil*nitrogen	<0.001

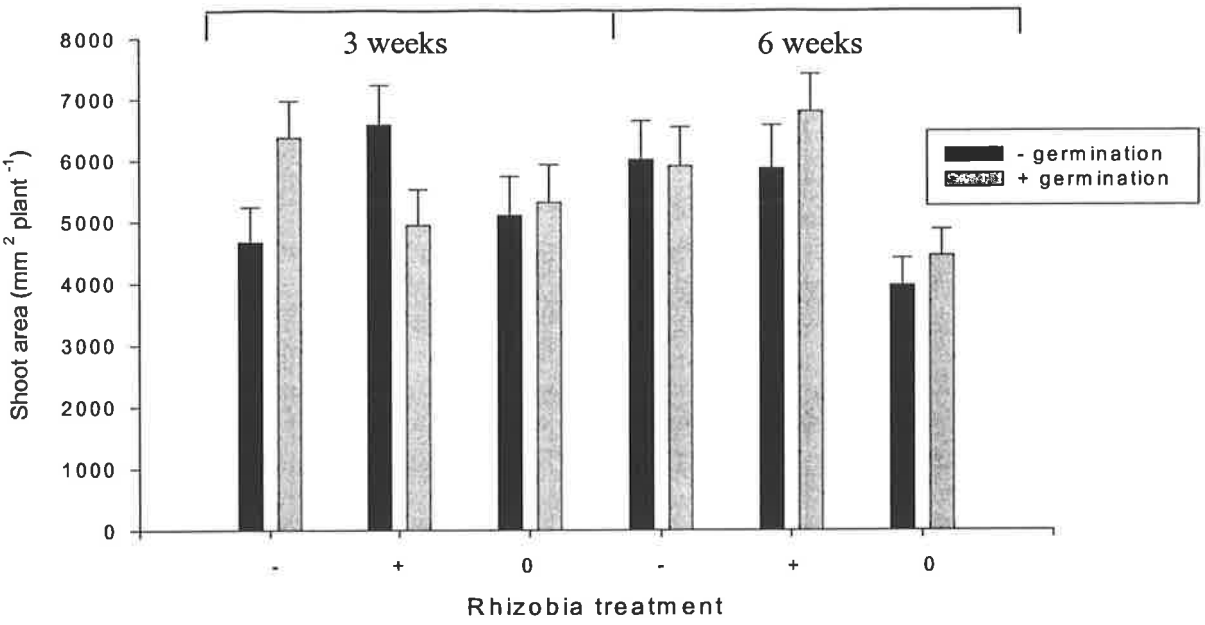


Figure 8.1: Effects of rhizobia (non pre-exposed (-), pre-exposed (+), not inoculated (0)) and germination (germinated in absence (-) and presence (+) of imazethapyr) treatments, and harvest time (3 and 6 weeks) on shoot area of chickpeas grown in a controlled environment room. Bars indicate standard error of mean.

The shoot area of chickpeas was affected by an interaction between nitrogen and harvest (Table 8.3; Figure 8.2). The addition of nitrogen increased shoot area by 30% at the second harvest (Figure 8.2). In the absence of nitrogen, the shoot area of chickpea plants decreased by 14% between the three and six week harvests, whilst in the presence of nitrogen, the shoot area of chickpea plants increased by 13% between the three and six week harvest (Figure 8.2).

Harvest time and the presence of imazethapyr in the soil interacted to affect the shoot area of chickpea plants (Table 8.3; Figure 8.3). At the first harvest, there was little difference in shoot area of chickpeas when imazethapyr was present or absent in the soil (Figure 8.3). At the second harvest, the presence of imazethapyr in the soil reduced the shoot area of chickpea plants by 50% compared to those grown without imazethapyr in the soil (Figure 8.3).

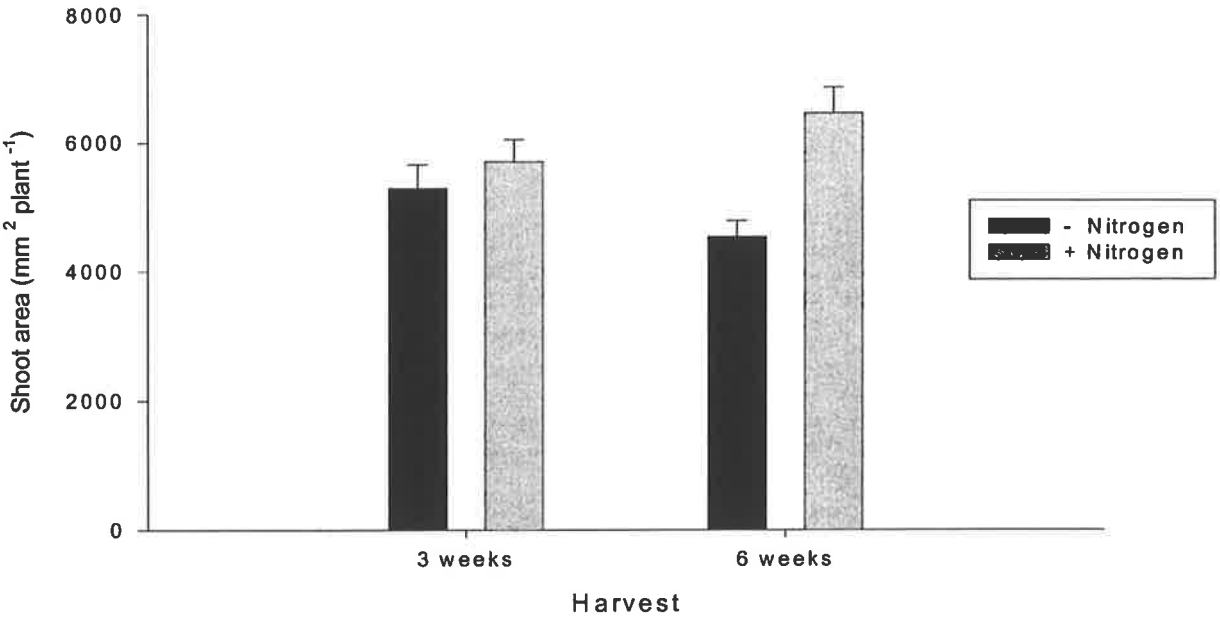


Figure 8.2: Effects of nitrogen fertiliser (+/-) and harvest time (3 and 6 weeks) on shoot area of chickpea plants grown in a controlled environment room. +/- indicates presence and absence of nitrogen fertiliser respectively. Bars indicate standard error of mean.

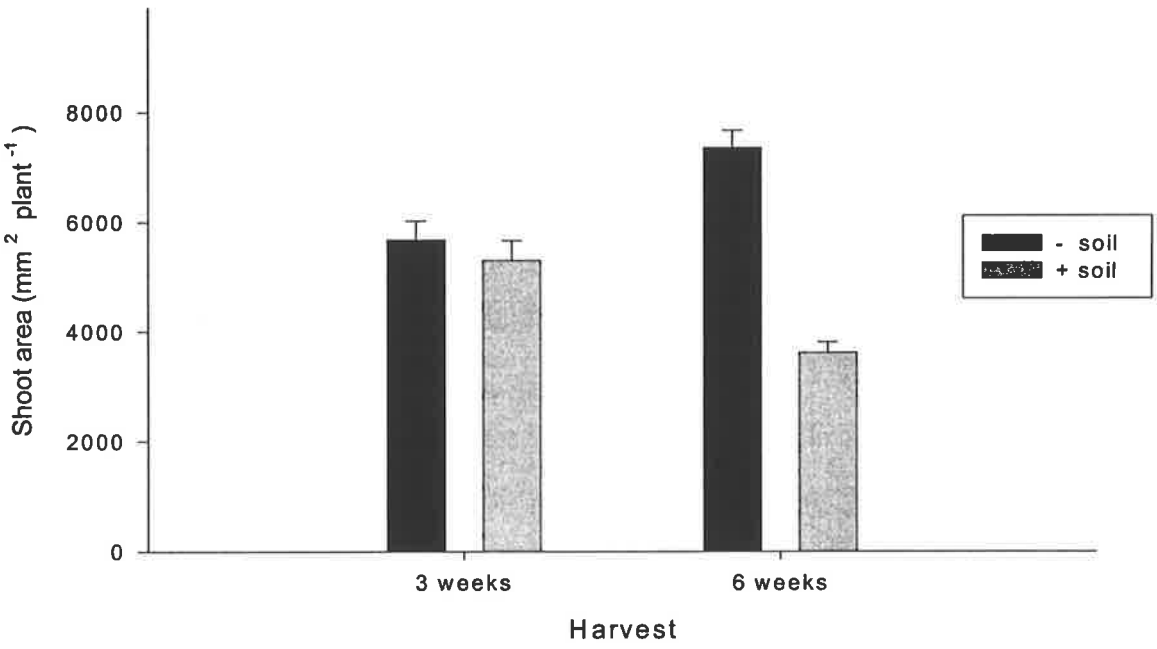


Figure 8.3: Effects of imazethapyr in soil and harvest time (3 and 6 weeks) on shoot area of chickpeas grown in a controlled environment room. +/- indicates presence and absence of imazethapyr respectively. Bars indicate standard error of mean.

The presence of imazethapyr in the soil interacted with nitrogen fertiliser to affect shoot area of chickpea plants (Table 8.3; Figure 8.4). In both the presence and absence of nitrogen fertiliser, the addition of imazethapyr to the soil decreased chickpea shoot area, however, the extent of reduction was 22% greater in the presence of nitrogen fertiliser (Figure 8.4).

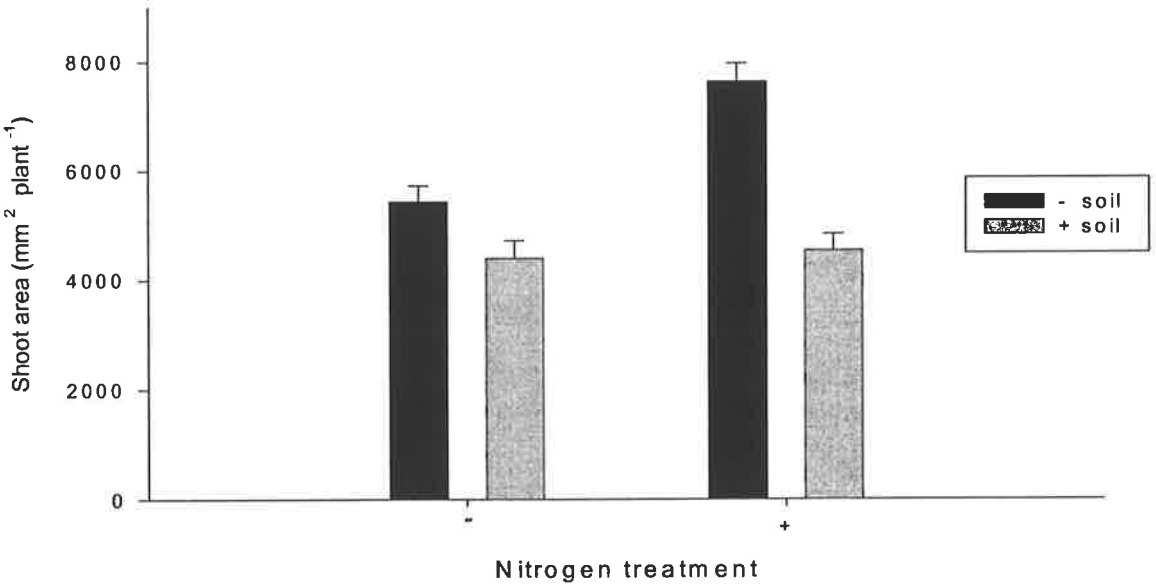


Figure 8.4: The effects of nitrogen fertiliser and the presence of imazethapyr in the soil on shoot area of chickpea plants grown in a controlled environment room. +/- Nitrogen and soil indicates presence or absence of nitrogen and imazethapyr from the soil respectively. Bars indicated standard error of mean.

8.3.2 Root biomass and root length density

Harvest time and the presence of imazethapyr in the soil interacted to affect root biomass (Table 8.4; Figure 8.5). The presence of imazethapyr in the soil reduced chickpea root biomass by approximately 60% at both harvests (Figure 8.5).

Table 8.4. Significant (α=0.05) effects of imazethapyr application on root biomass and root density of chickpea plants as determined by analysis of variance.

Variable	Source	P value
Root biomass	Soil*harvest	<0.001
	Soil*nitrogen	0.042
	Soil*rhizobia	0.037
Root length density (both harvests)	Germination*soil*nitrogen*harvest	0.022
Root length density (harvest 1)	Gemination*soil*nitrogen	0.043
Root length density (harvest 2)	Gemination*soil*nitrogen	0.011

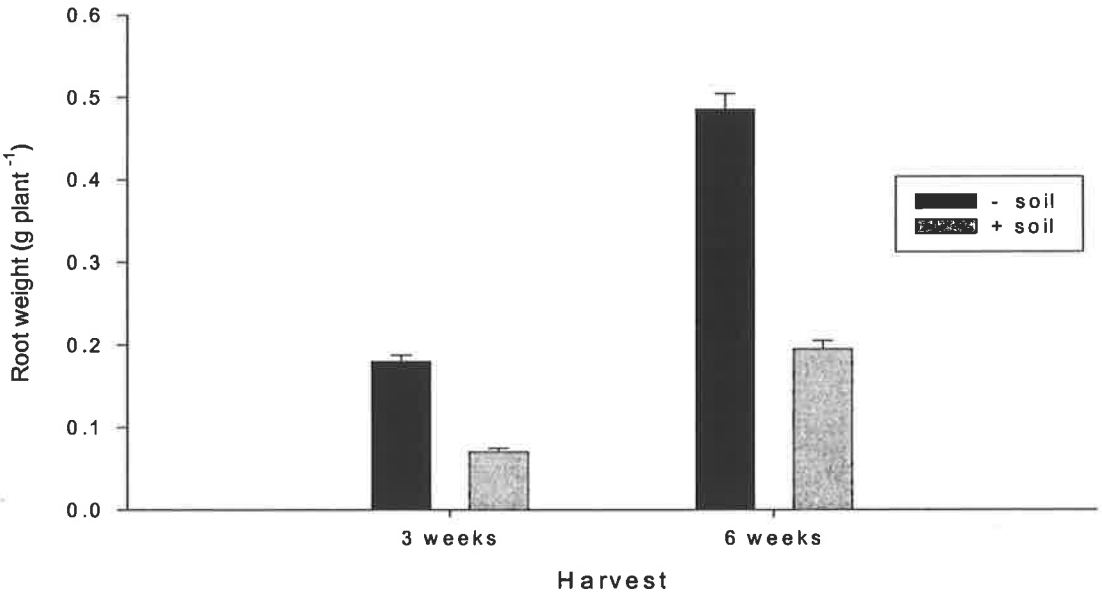


Figure 8.5: The effects of imazethapyr in the soil and harvest time (3 and 6 weeks) on root biomass of chickpea plants grown in a controlled environment room. +/- indicate presence or absence respectively of imazethapyr in the soil in which the plants were grown. Bars indicate standard error of mean.

Imazethapyr in the soil and nitrogen fertiliser interacted to affect the root biomass of chickpeas (Table 8.4; Figure 8.6). The presence of imazethapyr in the soil reduced the root biomass of chickpea plants both in the presence and absence of

nitrogen fertiliser (Figure 8.6). The magnitude of this reduction increased by 10% when nitrogen was present (Figure 8.6).

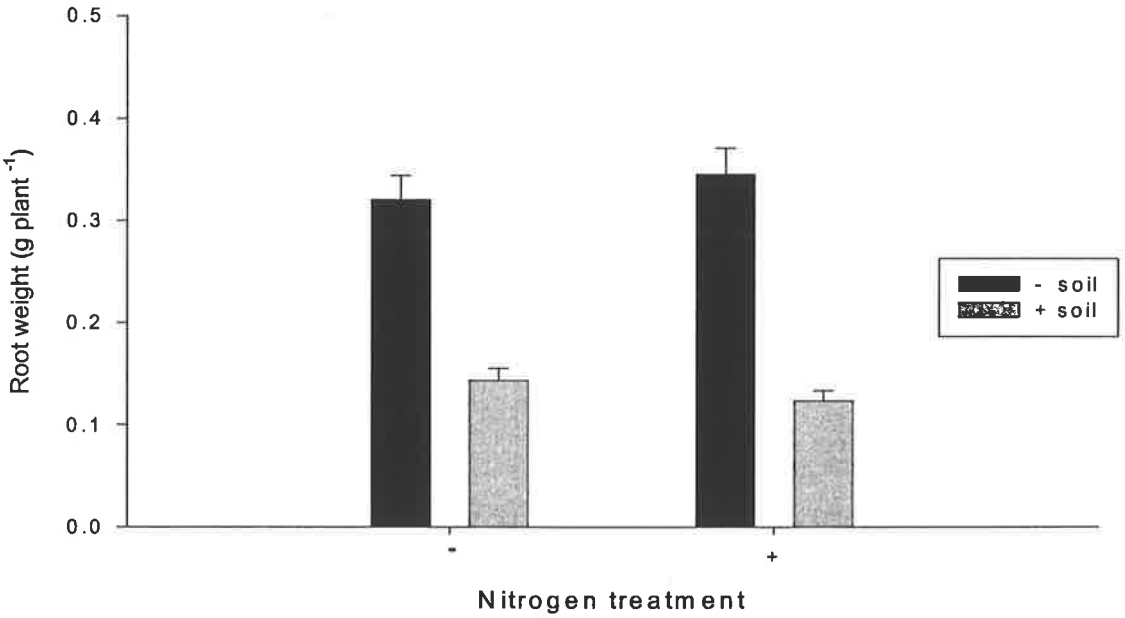


Figure 8.6: Effects of imazethapyr presence in soil and nitrogen fertiliser (+/-) on root biomass of chickpea plants grown in a controlled environment room. +/- nitrogen and soil indicates presence and absence of nitrogen or imazethapyr in the soil respectively. Bars indicate standard error of mean.

Rhizobia treatments and the presence of imazethapyr in the soil interacted to affect the root biomass of chickpea plants (Table 8.4; Figure 8.7). Under all rhizobia treatments, the presence of imazethapyr in the soil reduced root biomass by up to 65% (Figure 8.7). When imazethapyr was absent from the soil, pre-exposed rhizobia and non-inoculated treatments had a 13% lower root biomass than those that were inoculated with non pre-exposed rhizobia (Figure 8.7).

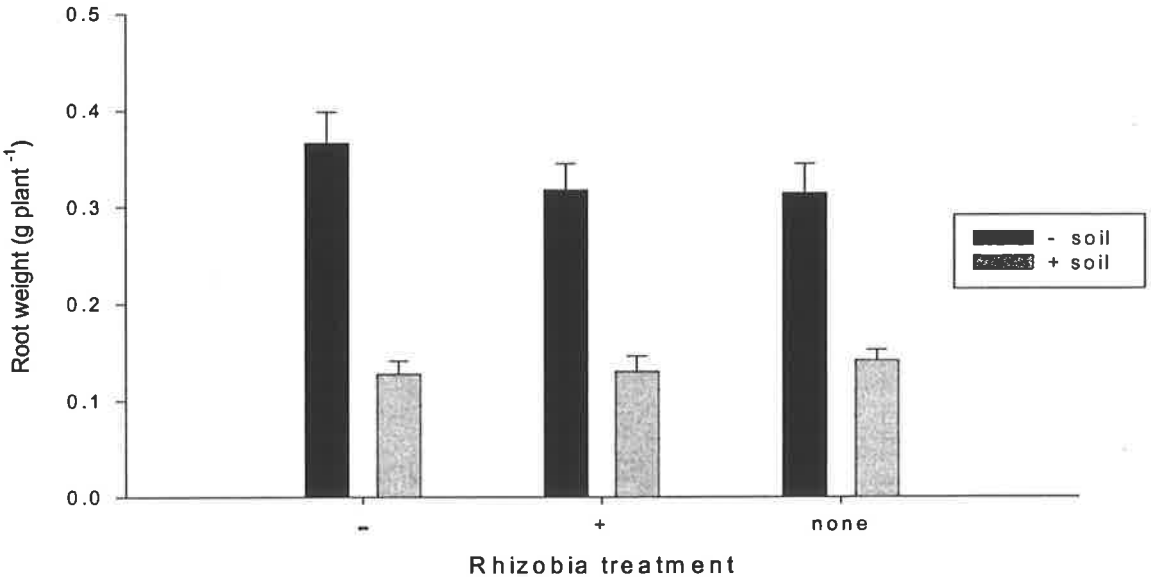


Figure 8.7: The effects of rhizobia treatments and the presence of imazethapyr in the soil on root biomass of chickpea plants grown in a controlled environment room. -/+/none along the x-axis indicate rhizobia not pre-exposed (-) or pre-exposed (+) to imazethapyr and not inoculated treatments. +/- soil indicate the presence or absence of imazethapyr in the soil. Bars indicate standard error of mean.

Root length density of chickpea roots was affected by a four-way interaction between germination treatment, the presence of imazethapyr in the soil, nitrogen fertiliser and harvest time (Table 8.4; Figure 8.8). As with the chlorsulfuron experiment, the data were split at the harvest level and then the two harvests re-analysed separately by analysis of variance to help explain the four-way interaction (Table 8.4).

At the first harvest, root length density was affected by an interaction between soil, germination and nitrogen treatments (Table 8.4). The presence of imazethapyr in the soil reduced the root length density of chickpea plants, regardless of germination or nitrogen treatments (Figure 8.9). When imazethapyr was present at the time of

germination and absent from the soil, the addition of nitrogen fertiliser increased the root length density of chickpea plants by 17% (Figure 8.9). However, when imazethapyr was absent at both germination and from the soil, the addition of nitrogen fertiliser decreased root length density of chickpea plants by 10% (Figure 8.9).

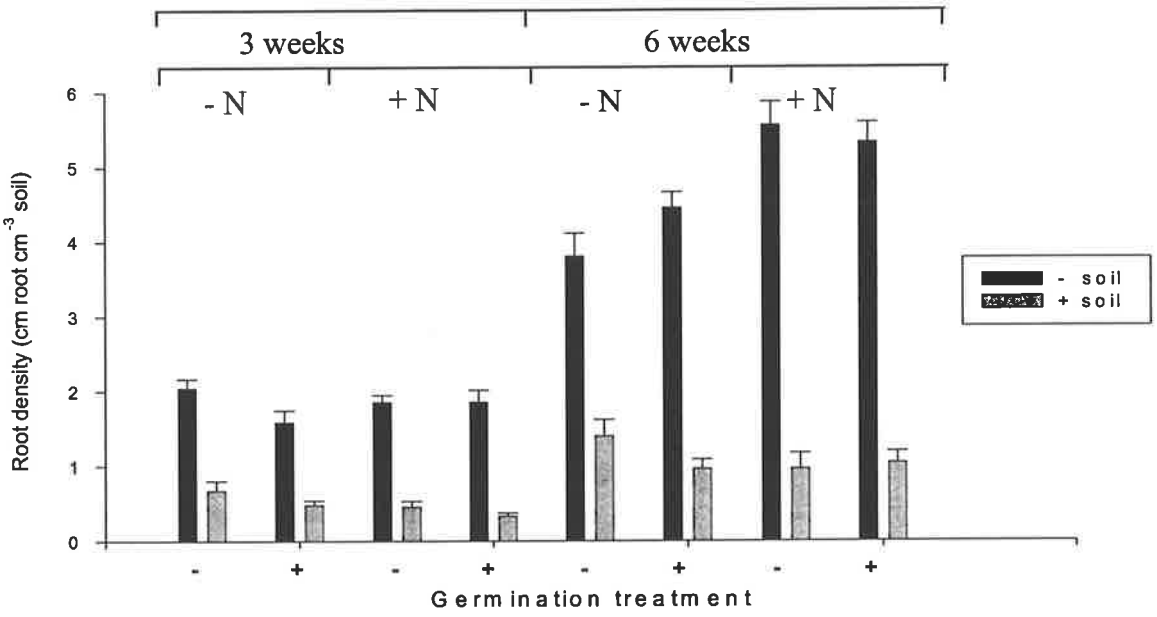


Figure 8.8: Effects of germination in presence of imazethapyr, presence of imazethapyr in the soil, nitrogen fertiliser and harvest time (3 and 6 weeks) on root length density of chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

At the second harvest, the root length density of chickpeas was again affected by an interaction between soil, germination and nitrogen treatments (Table 8.4). After six weeks of growth, the presence of imazethapyr in the soil continued to reduce the root length density of chickpea plants (Figure 8.10). The addition of nitrogen fertiliser increased root density by 46% when imazethapyr was absent from the soil and at germination (Figure 8.10). The presence of imazethapyr at germination resulted in an increase in root length density of 17% in the absence of nitrogen and imazethapyr in the

soil, compared to those treatments germinated in the absence of imazethapyr (Figure 8.10). When nitrogen was present and imazethapyr absent from the soil, root length density was similar both in the presence and absence of imazethapyr at germination (Figure 8.10).

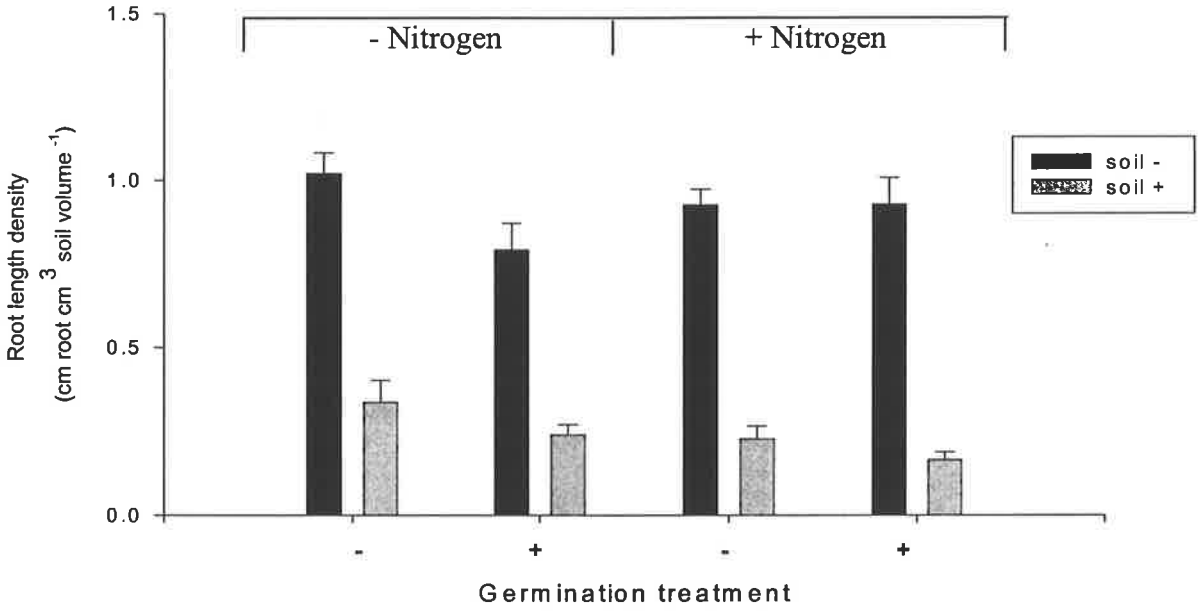


Figure 8.9: Effects of imazethapyr presence at germination and in the soil and nitrogen fertiliser (presence (+) or absence (-) on root length density of chickpeas at harvest one (three weeks after inoculation). Bars indicate standard error of mean. +/- soil or germination= presence/absence of imazethapyr respectively.

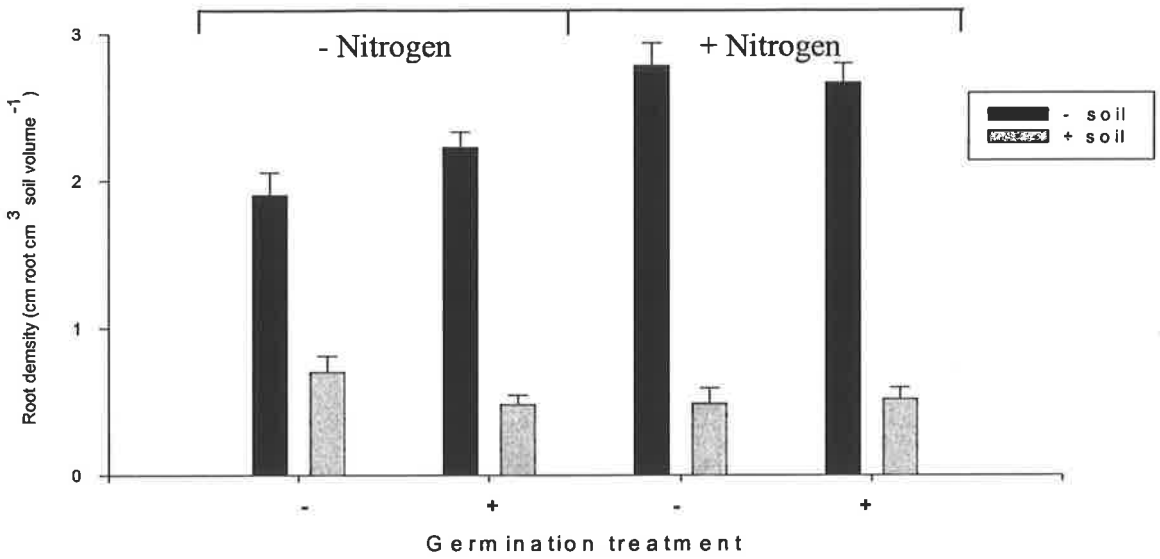


Figure 8.10: The effects of imazethapyr at germination and in the soil and nitrogen fertiliser (presence (+) or absence (-) on root length density of chickpea plants at harvest two (six weeks after inoculation). +/- soil or germination= presence/ absence of imazethapyr respectively. Bars indicate standard error of mean.

8.3.3 Number and weight of nodules

Number and weight of nodules refer to total number and weight of nodules on each chickpea plant. The presence of imazethapyr in the soil, nitrogen fertiliser and harvest time interacted to affect the number of nodules on chickpea plants (Table 8.5). The presence of imazethapyr in the soil, in the absence of nitrogen, reduced the number of nodules by 89% at both harvests. Nitrogen fertiliser, in the absence of chlorsulfuron, reduced the number of nodules by 23% at the first harvest and 41% at the second harvest (Figure 8.11).

Table 8.5: Significant ($\alpha = 0.05$) effects of imazethapyr application on the number of nodules and weight of nodules on chickpea plants as determined by analysis of variance.

Variable	Source	P value
Number of nodules	Soil*nitrogen*harvest	<0.001
	Rhizobia*harvest	0.035
	Rhizobia*soil	0.010
Nodule fresh weight	Soil*nitrogen	<0.001

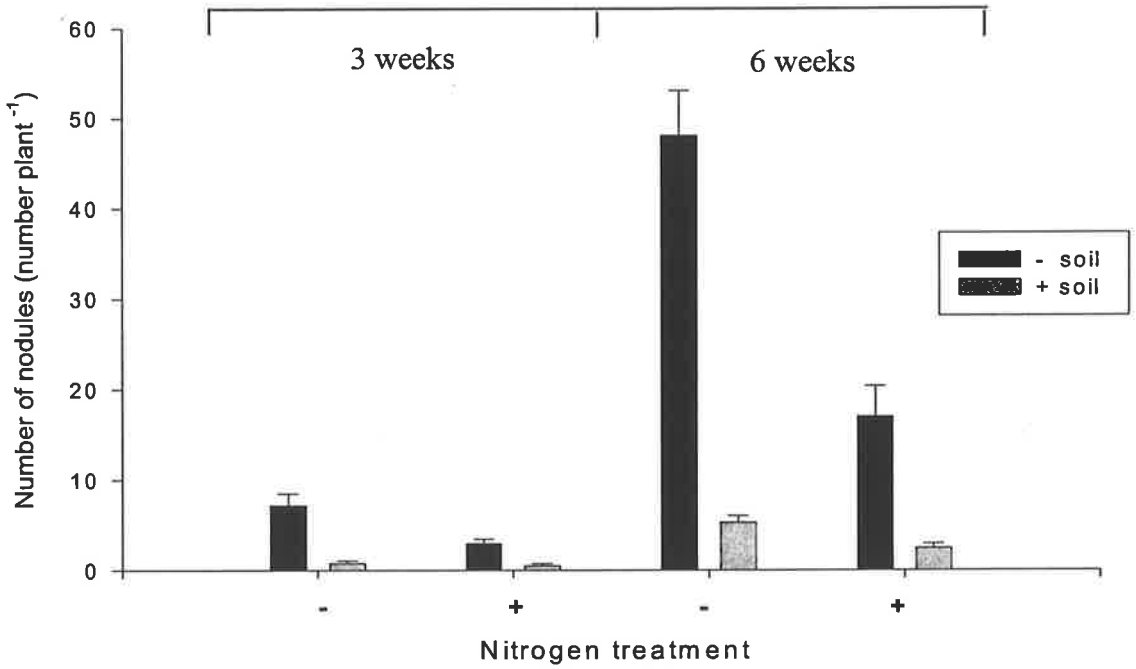


Figure 8.11: The effects of the presence of imazethapyr in the soil, nitrogen fertiliser and harvest time on number of nodules found on chickpea plants. +/- nitrogen or soil indicates presence or absence of nitrogen or soil respectively. Bars indicate standard error of mean.

Rhizobia treatments and harvest time interacted to affect the number of nodules on chickpeas (Table 8.5). At the six week harvest, the number of nodules was reduced by 33% when rhizobia were pre-exposed to imazethapyr prior to inoculation (Figure

8.12). At the first harvest, the difference in number of nodules between pre-exposed and non-pre-exposed rhizobia treatments was 26% (Figure 8.12).

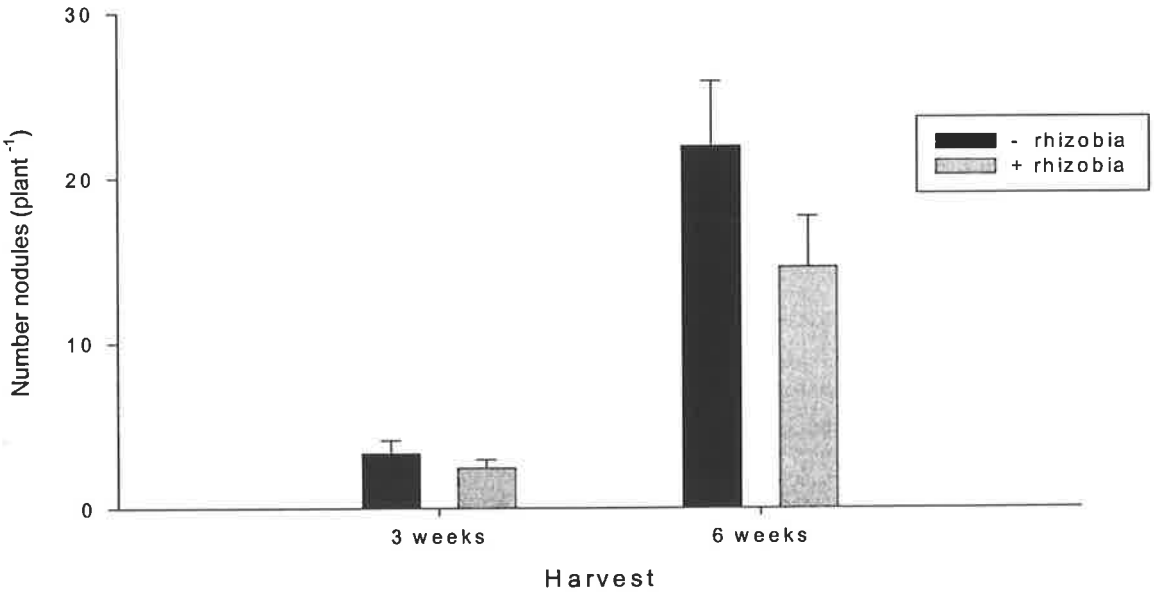


Figure 8.12: The effects of rhizobia treatment (pre-exposed (+) and non pre-exposed (-)) and harvest time (3 and 6 weeks) on number of nodules on chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

Rhizobia treatments and the presence of imazethapyr in soil interacted to affect the number of nodules on chickpea plants (Table 8.5). When chickpea plants were grown with imazethapyr in the soil, the number of nodules was reduced by 90% compared to those grown without herbicide in the soil and during the growth of rhizobia. But, in the presence of imazethapyr in the soil, there was no difference between the number of nodules of treatments that were inoculated with rhizobia either pre-exposed or not pre-exposed to imazethapyr prior to inoculation (Figure 8.13). However, in the absence of imazethapyr in the soil, inoculation with pre-exposed

rhizobia resulted in a 35% reduction in the nodules compared to those that were inoculated with non-pre-exposed rhizobia. (Figure 8.13).

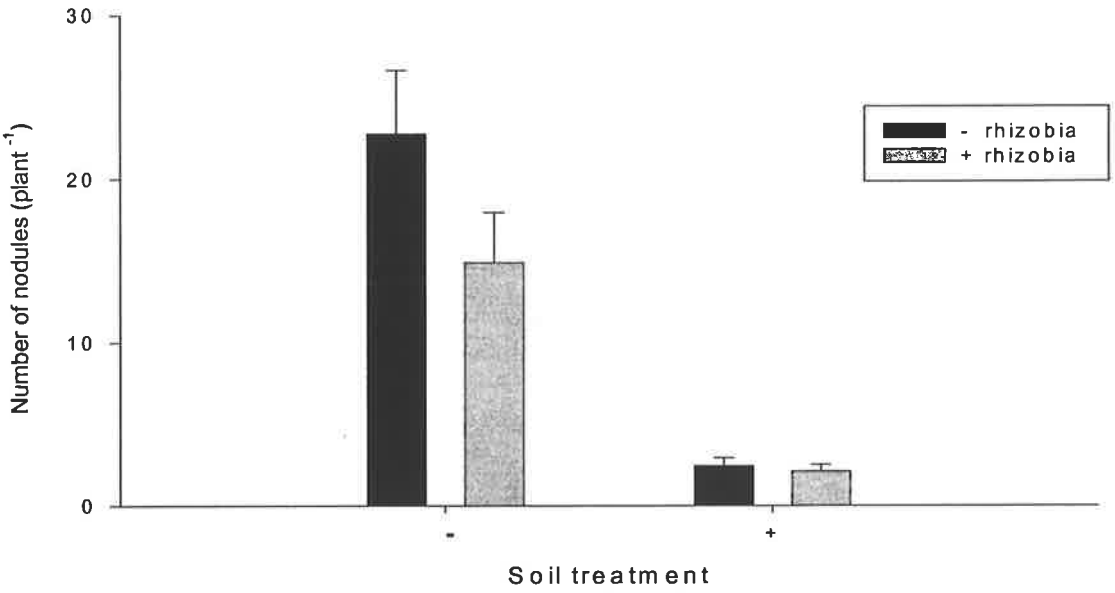


Figure 8.13: The effects of rhizobia treatments (pre-exposed (+) and non pre-exposed (-)) and presence of imazethapyr in soil on the number of nodules on chickpea plants grown in a controlled environment room. Bars indicate standard error of mean.

Chickpea nodule weight (g plant^{-1}) was affected by an interaction between nitrogen fertiliser and presence of imazethapyr in the soil (Table 8.5). The presence of nitrogen fertiliser reduced the weight of nodules both in the presence (88%) and absence (70%) of imazethapyr in the soil (Figure 8.14). In the absence of nitrogen fertiliser, the presence of imazethapyr in the soil resulted in a 63% reduction in nodule weight compared to those grown in soils without imazethapyr (Figure 8.14).

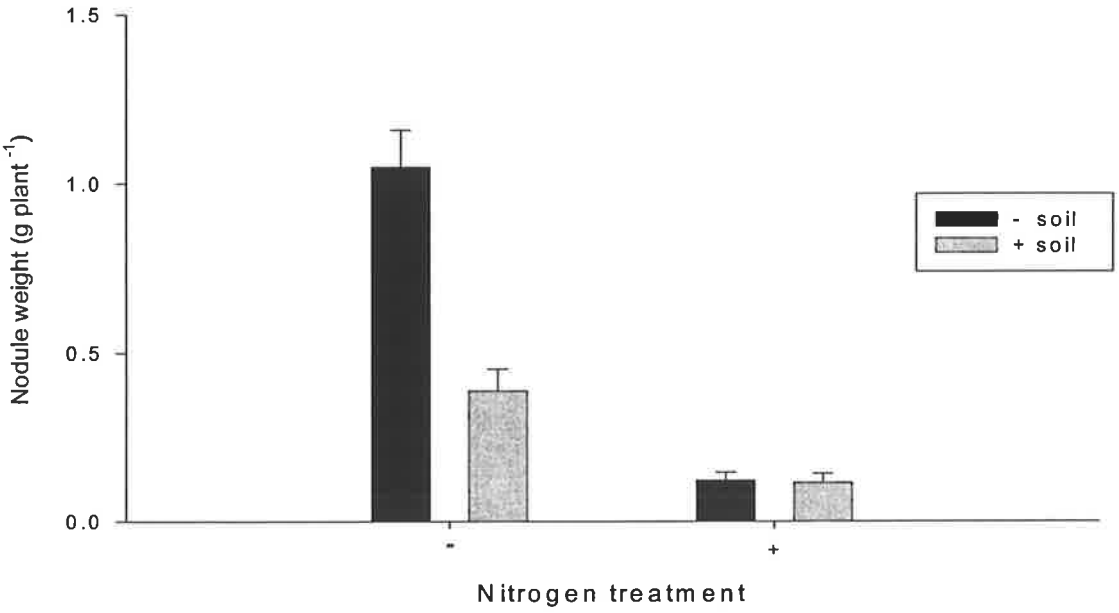


Figure 8.14: The effects of nitrogen fertiliser (presence (+) or absence (-)) and presence of imazethapyr in the soil on nodule weight of chickpea plants six weeks after inoculation. Bars indicate standard error of mean.

A linear regression analysis was performed on all of the data to determine if there was a relationship between total weight and number of nodules on each chickpea plant and to investigate changes in slopes and intercepts of the lines to indicate that the relationship varies between the applied treatments. The data set was separated based on nitrogen treatment, for the same reasons discussed in Chapter 7 (Section 7.3.4). Positive relationships between nodule weight and number of nodules were obtained in both the absence ($p< 0.0001$; Figure 8.15) and presence ($p=0.0076$; Figure 8.16) of fertiliser nitrogen. However, in the presence of nitrogen the relationship was less well defined ($r^2 = 0.1731$). In the absence of nitrogen, the data was split on the basis of rhizobia treatment (Table 8.6). In the absence of nitrogen, positive significant regressions were found for both non pre-exposed rhizobia ($p<0.0001$) and pre-exposed rhizobia ($p<0.0001$) treatments (Table 8.6).

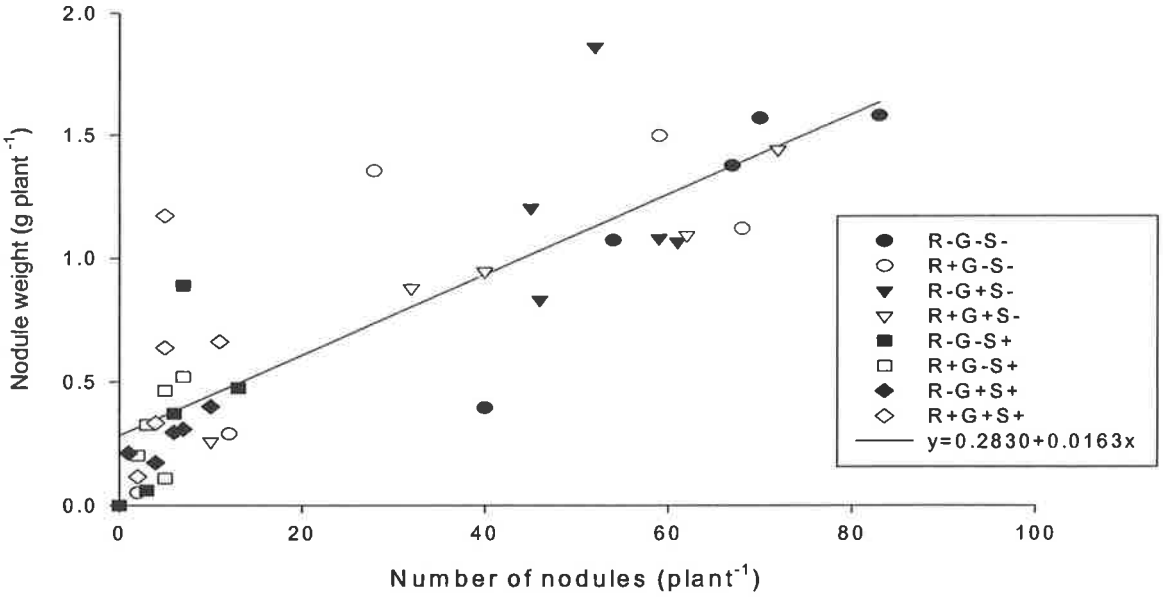


Figure 8.15: Relationship between nodule number and total nodule weight of chickpea plants grown in the absence of additional nitrogen fertiliser, six weeks after inoculation (r^2=0.71; p<0.0001). R = rhizobia treatment; G = germination treatment; S = soil treatment. +/- = presence and absence of chlorsulfuron respectively in each treatment.

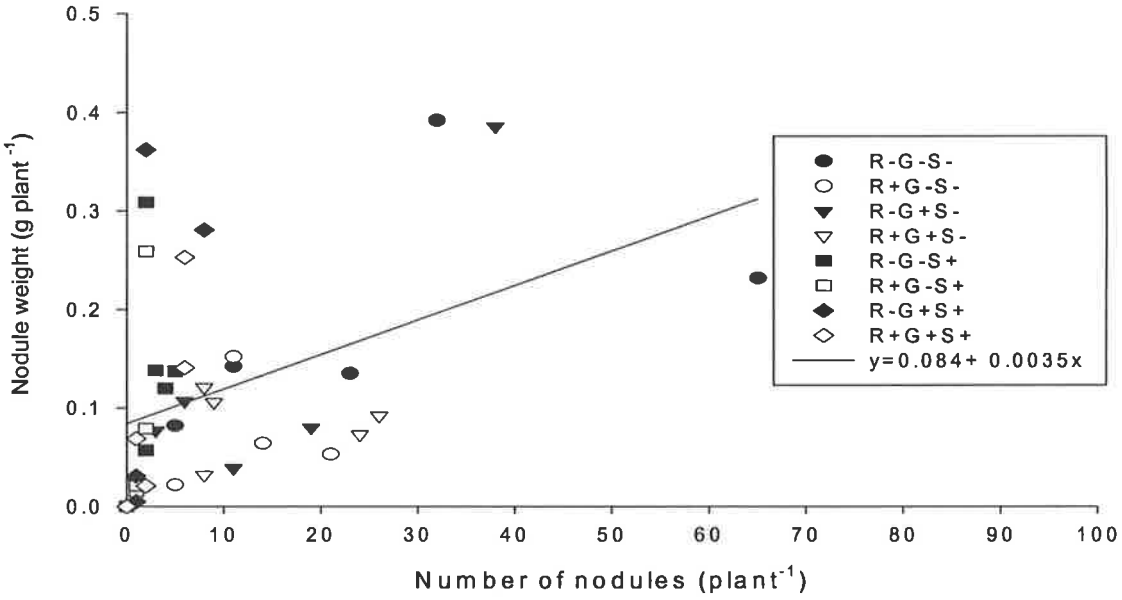


Figure 8.16: Relationship between nodule number and total nodule weight of chickpea plants grown in the presence of additional nitrogen fertiliser, six weeks after inoculation (r^2=0.17; p=0.0076). R = rhizobia treatment; G = germination treatment; S = soil treatment. +/- = presence and absence of chlorsulfuron respectively in each treatment.

Table 8.6: Regression table for nodule weight against number of nodules for chickpea plants grown in the absence of nitrogen and separated on the basis of rhizobia treatment (pre-exposed and non pre-exposed to imazethapyr) ($\alpha = 0.05$). Standard errors of slopes and intercepts are displayed in parentheses.

Treatment		Slope	Intercept	r^2	P value
No additional nitrogen	(i) All treatments (ie +/- rhizobia and soil)	0.016 (0.002)	0.283 (0.064)	0.71	<0.0001
	(ii) - Rhizobia	0.018 (0.002)	0.207 (0.095)	0.77	<0.0001
	+ Rhizobia	0.015 (0.002)	0.341 (0.089)	0.64	<0.0001

8.3.4 Total nitrogen or plant nitrogen

The plant nitrogen of chickpea roots and shoots (combined) was significantly affected by a two way interaction between nitrogen fertiliser and the presence of imazethapyr in the soil (Table 8.7). The addition of nitrogen fertiliser increased nitrogen content of chickpea plants by 117% in the absence, and only 53% in the presence, of imazethapyr in the soil (Figure 8.17).

Table 8.7. Significant (α=0.05) effects of imazethapyr applications on plant (shoot and roots combined) nitrogen of chickpeas as determined by analysis of variance.

Variable	Source	P value
Total Nitrogen (plant nitrogen)	Soil*nitrogen	<0.001
	Rhizobia *soil	<0.001
Nitrogen fixed (inference)	Soil	<0.001

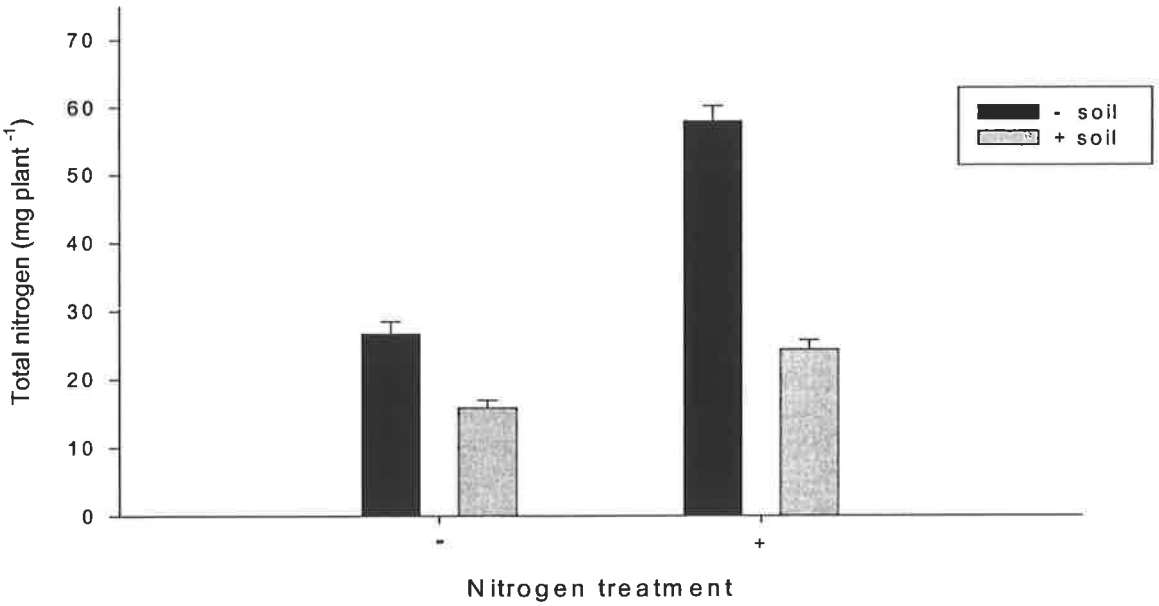


Figure 8.17: The effects of nitrogen fertiliser (presence (+) or absence (-)) and presence of imazethapyr in the soil on plant nitrogen of chickpeas grown in a controlled environment room. Bars indicate standard error of mean.

There was a significant interaction between pre-exposure of rhizobia and presence of imazethapyr in soil, on chickpea plant nitrogen (Table 8.7). The presence of imazethapyr reduced plant nitrogen regardless of rhizobia treatment (Figure 8.18). When imazethapyr was absent from the soil there was little difference in plant nitrogen between the pre-exposed and non pre-exposed rhizobia treatments (Figure 8.18). When imazethapyr was present in the soil, plant nitrogen was reduced by 60% when rhizobia were not pre-exposed, 51% when rhizobia were pre-exposed to imazethapyr, and 43% when the plants were not inoculated compared to plants grown without imazethapyr present in the soil (Figure 8.18).

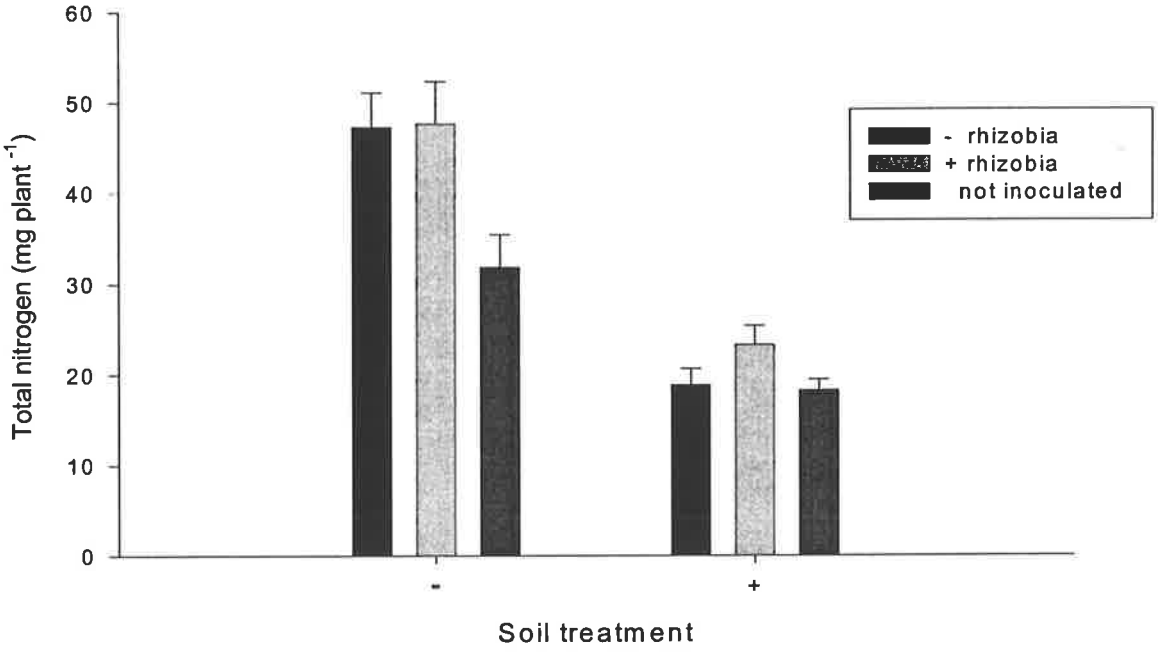


Figure 8.18: The effects of the presence of imazethapyr in soil and during the growth of rhizobia on plant nitrogen of chickpeas grown in a controlled environment room. +/- indicates presence or absence of imazethapyr respectively. Bars indicate standard error of mean.

There was a significant main effect of presence of imazethapyr on estimated fixed nitrogen by inoculated chickpea plants grown in the absence of nitrogen (Table 8.7). The presence of imazethapyr in the soil reduced the plant nitrogen of chickpeas from 13.993 mg plant^{-1} to 0.628 mg plant^{-1} (96% reduction). It can therefore be inferred that the amount of nitrogen fixed by inoculated chickpea plants, grown in the absence of nitrogen fertiliser, was reduced by this same amount (96%).

8.3.5 Relationship between plant nitrogen and nodule weight

As in Chapter 7, regression analyses were used to determine the relationship between plant nitrogen and nodule weight, six weeks after inoculation and to compare slopes and intercepts to investigate the difference in treatments. This provided an

indication of the efficiency of the nodules. The data acquired for the two nitrogen fertiliser treatments were analysed separately (Table 8.8), because nitrogen interacted with soil and rhizobia treatments to affect the plant nitrogen of chickpeas. A significant positive regression relating increasing nodule weights to plant nitrogen was obtained when the plants were grown in the absence of additional nitrogen ($p < 0.0001$; Figure 8.19). However, the regression between nodule weight and plant nitrogen was not significant when plants were grown in the presence of additional nitrogen ($p = 0.2695$).

The data was then split based on rhizobia treatment due to interaction effects on plant nitrogen observed in section 8.3.5 (Table 8.8). In the absence of nitrogen, positive significant regressions of nodule weight against plant nitrogen were found for non pre-exposed ($p<0.0001$; Table 8.8) and pre-exposed rhizobia treatments ($p<0.0001$; Table 8.8). In the presence of nitrogen however, the regressions of nodule weight versus plant nitrogen were both non-significant (Table 8.8).

The data was also split based on soil treatment due to interaction effects on plant nitrogen observed in section 8.3.5 (Table 8.8). In the absence of nitrogen, there was a significant regression between nodule weight and plant nitrogen of chickpeas for both plants grown without imazethapyr in the soil ($p<0.0001$; Table 8.8) and with imazethapyr in the soil ($p=0.0002$; Table 8.8). In the presence of additional nitrogen however, the regression of nodule weight and plant nitrogen was significant only for plants grown with imazethapyr in the soil ($p= 0.0489$; Table 8.8).

Table 8.8: Regression table for nodule weight against plant nitrogen for chickpea plants. i) separated on the basis of nitrogen treatment with all other treatments included; ii) separated on the basis of rhizobia treatment (pre-exposed and non pre-exposed to imazethapyr ; iii) separated on the basis of soil treatment (Presence (+) and absence (-) of imazethapyr in the soil. (α= 0.05). Standard errors of slopes and intercepts are displayed in parentheses.

Treatment		Slope	Intercept	r^2	P value
No additional nitrogen	(i) All treatments (ie +/- rhizobia and soil)	19.034 (1.532)	-0.496 (1.350)	0.80	<0.0001
	(ii) - Rhizobia	20.023 (2.028)	-2.489 (1.905)	0.84	<0.0001
	+ Rhizobia	18.155 (2.303)	1.337 (1.887)	0.78	<0.0001
	(iii) – Soil	15.992 (2.337)	4.012 (2.690)	0.72	<0.0001
	+ Soil	16.682 (3.606)	-0.903 (1.724)	0.54	0.0002
Additional nitrogen	(i) All treatments (ie +/- rhizobia and soil)	34.331 (30.639)	30.204 (4.919)	0.03	0.2695
	(ii) - Rhizobia	55.541 (36.264)	23.593 (7.223)	0.12	0.1430
	+ Rhizobia	20.367 (68.001)	34.630 (7.409)	0.05	0.7680
	(iii) – Soil	26.444 (21.527)	49.852 (3.413)	0.08	0.2351
	+ Soil	31.664 (14.991)	11.822 (2.436)	0.20	0.0489

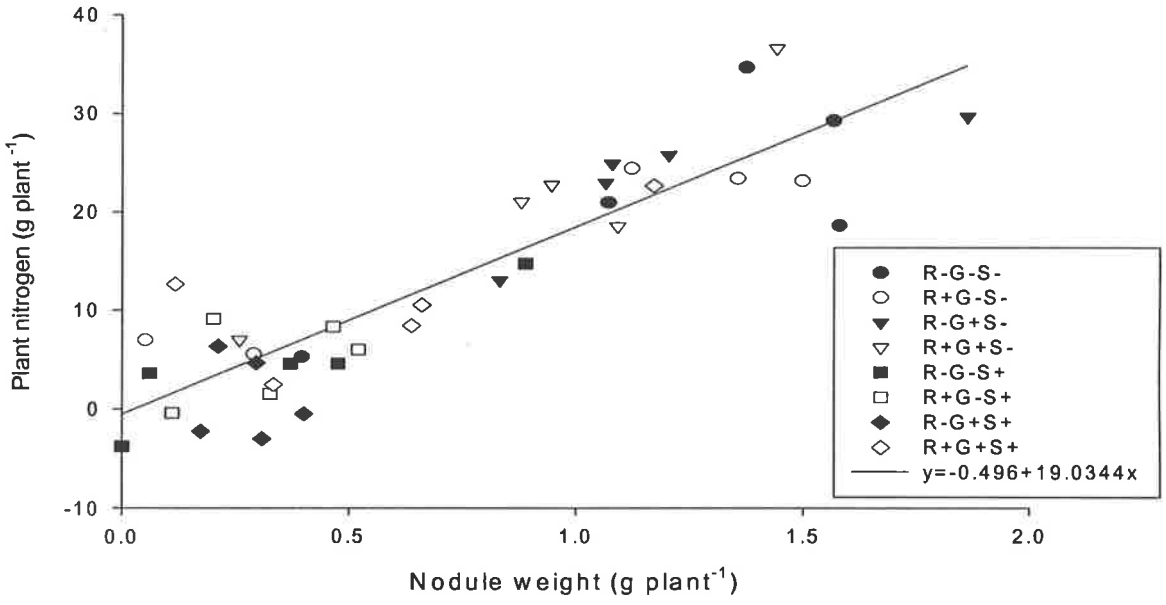


Figure 8.19: Relationship between nodule weight and plant nitrogen of chickpeas grown in the absence of additional nitrogen fertiliser, six weeks after rhizobial inoculation. (r2=0.80; p<0.0001). R= rhizobia treatment; G = germination treatment; S = soil treatment; +/- = presence and absence of imazethapyr respectively.

8.4 DISCUSSION

8.4.1 Effects of imazethapyr on plant biomass, shoot area and root length density of chickpeas grown under controlled environmental conditions

Although shoot biomass was not reduced, chickpea shoot area, root biomass, root length density and nodulation were all reduced when imazethapyr was present in the soil. However, the reductions in root biomass and shoot area observed after application of imazethapyr, suggest it is likely that had the experiment continued, a reduction in chickpea shoot biomass would have been observed. Chickpea roots had the initial contact with the herbicide and, therefore, exhibited the first symptoms. The herbicide would be translocated to the shoots through the xylem or phloem and cell division would cease in these regions, unless the plant is able to metabolically inactivate the herbicide before it reaches its target site (Brown, 1990; Beyer *et al.*, 1988). As imazethapyr is recommended for use in chickpeas (Spinnaker herbicide label), it can be expected that the plant would be tolerant and, therefore, able to metabolically inactivate the herbicide. However, most of the results from this study suggest that chickpeas were not tolerant to imazethapyr.

If pre-exposing rhizobia reduced nodulation, it is possible that nitrogen fixation would be reduced and that in turn shoot area would be reduced. In fact, rhizobia, germination and harvest times interacted to affect shoot area. This does not imply that pre-exposing rhizobia would affect shoot area of chickpeas, but rather that the rhizobia treatments responded differently to the germination treatments at each harvest time. The interaction was due to the shoot area of plants inoculated with non pre-exposed rhizobia treatments increasing in the presence of herbicide at germination at the first

harvest, while pre-exposed rhizobia treatments decreased in shoot area in the presence of herbicide at germination. All of the other treatments were consistent across both harvest times, with little difference between presence and absence of imazethapyr at germination. By the six week harvest there was no longer any difference between presence and absence at germination for non pre-exposed rhizobia, suggesting that any initial effects observed (i.e. increase in shoot area from pre germinating seeds in the presence of imazethapyr) were short lived. In the absence of nitrogen, shoot area of chickpeas decreased between 3 and 6 weeks, suggesting that the plants are nitrogen limited.

Imazethapyr reduced shoot area of chickpeas but not shoot biomass. Shoot area was measured because it was observed that leaves were smaller on affected plants, but this was not reflected in shoot biomass. The morphology of two leaf components, rachis and leaflet, is very sensitive to environmental conditions and can change drastically (Cubero, 1987). The herbicide caused stress to the plants, limiting the size of the leaves and leaflets and if the experiment had continued then greater differences would probably have been observed in shoot biomass as well as shoot area. This was also observed in the field experiment in Chapter 5, where imazethapyr reduced both leaf area index and shoot biomass at flowering.

Decreased chickpea root biomass due to the application of imazethapyr is supported by results from other authors. Imazethapyr applied at rates that ranged from 0.7 to 14 times the recommended application rate reduced the biomass of pea (*Pisum sativum*) roots by up to 60% compared to control plants (Gonzalez *et al.*, 1996). Increasing the concentration of imazethapyr from 0 to 14 times the recommended application rate resulted in a reduction in root length of approximately 75% at the

highest concentration in peas (Gonzalez *et al.*, 1996). Results obtained from the lower concentrations by Gonzalez *et al.* (1996), are comparable to the results from this trial where the recommended application rate of imazethapyr was used. At the recommended application rate of imazethapyr for peas, Gonzalez *et al.* (1996) found a root and shoot biomass reduction of approximately 10%. The recommended application rate for peas (50 – 100 g ai ha^{-1}) (Hart *et al.*, 1991) is approximately 2 – 4 times that for chickpeas (29 g ai ha^{-1}) (Cyanamid herbicide label). In the study discussed in this chapter, chickpea shoot weight was not affected, but root biomass was reduced by up to 60%. The greater reduction in root biomass observed in this chapter may be due to a greater sensitivity of chickpeas than peas to imazethapyr.

The addition of nitrogen fertiliser did not alleviate the imazethapyr symptoms observed in shoot area, root biomass or root length density. This is probably due to imazethapyr reducing root biomass and root length density with less nitrogen fertiliser taken into the plant.

The presence of imazethapyr in the soil, caused the greatest reductions in root biomass, and probably masked any effects that may have been observed from imazethapyr presence during the growth of rhizobia or at germination. For example, when imazethapyr was present in the soil there was no difference between the root biomass of any rhizobia treatment. However, when imazethapyr was absent from the soil, pre-exposed rhizobia and non-inoculated plants had lower root biomass than non pre-exposed rhizobia treatments.

8.4.2 Effects of imazethapyr on nodulation and plant nitrogen of chickpeas grown under controlled environment conditions

Imazethapyr reduced the number and weight of nodules on chickpea roots. The greatest reductions in nodulation were observed when imazethapyr was present in the soil in which the plants were grown, and was likely a reflection of reduced root biomass and root length density, leading to fewer infection sites. Nitrogen fertiliser also reduced the number of chickpea nodules in this study, and inorganic nitrogen has been shown to inhibit the nodulation of chickpeas (Jessop *et al.*, 1984).

The number, but not weight, of nodules was reduced in plants inoculated with rhizobia pre-exposed to imazethapyr. This suggests that the herbicide is reducing the number of infections rather than the size of the nodules. It is possible that the plant is placing more resources into creating larger nodules than forming new ones in the adverse conditions created by herbicide damage (Rupela and Saxena, 1987). The relationship between nodule weight and nodule number supports this to an extent, although the data were variable. The majority of plants inoculated with non pre-exposed rhizobia (in the absence of nitrogen and imazethapyr in the soil) had high nodule weights and numbers. However, the data from plants inoculated with pre-exposed rhizobia were highly variable with data from individual plants scattered along the nodule weight by nodule number regression line. From the data it was difficult to determine whether the plants inoculated with pre-exposed rhizobia are producing a few larger nodules. In the absence of nitrogen, there was no difference in the slopes of nodule number and nodule weight regressions, between pre-exposed and non pre-exposed rhizobia treatments, suggesting that the nodules of both treatments were similar in size. These results were similar to those found by Gonzalez *et al.* (1996), where imazethapyr had a greater impact on nodulation than on pea growth, although rhizobial

growth was not affected. At 0.7 times the recommended application rate, imazethapyr reduced the number of nodules on pea plants by approximately 10% and this reduction increased to approximately 45% in the presence of 3.5 times the recommended application rate. Gonzalez *et al.* (1996), found that nodule number was affected more than nodule weight, suggesting a direct impact on the nodule initiation rather than on nodule development (Gonzalez *et al.*, 1996), as is also suggested by the results discussed in this chapter. Inhibition of nodulation has been reported for other herbicides (e.g. atrazine on crownvetch) and is thought to be related to toxic effects on the plant rather than to effects on microorganisms (Cardina *et al.*, 1986; Rennie and Dubetz; 1984).

When inoculated chickpeas were grown in the absence of nitrogen (in this study), imazethapyr reduced plant nitrogen inferring that the herbicide reduced the amount of nitrogen fixed. The addition of nitrogen fertiliser, doubled the slope of the plant nitrogen on nodule weight regression compared to those plants grown without nitrogen. However, the regression with nitrogen fertiliser was not significant and will not be discussed further. In the absence of nitrogen, the slopes of the plant nitrogen on nodule weight regressions for both rhizobia pre-exposed and not pre-exposed to imazethapyr were similar. The similarity of slopes suggests that plants inoculated with pre-exposed and non pre-exposed rhizobia produced the same amount of nitrogen per unit of nodule weight. The same was observed with the slopes of the plant nitrogen on nodule weight regression for plants grown in soil with and without imazethapyr, which were again similar. The similarity of slopes suggests that the presence of imazethapyr in the growth medium, is not affecting the amount of nitrogen produced per unit of nodule weight.

Imazethapyr in the soil reduced the total plant nitrogen of chickpeas and the addition of nitrogen fertiliser did not alleviate the reduction. It appeared that the presence of nitrogen fertiliser resulted in a greater reduction in chickpea plant nitrogen in the presence of imazethapyr in the soil, compared to those plants grown without imazethapyr in the soil (Figure 8.18). But this apparent greater reduction was more likely due to the plants grown without imazethapyr in the soil, responding more to nitrogen fertiliser due to greater uptake ability through increased root biomass, while the plants grown with imazethapyr in the soil were incapable of taking up the additional available nitrogen from the soil due to reduced root biomass.

8.4.3 Comparison of effects of chlorsulfuron (Chapter 7) and imazethapyr (Chapter 8) on growth and biomass, of chickpea plants grown in pots, under controlled conditions.

Both chlorsulfuron (Chapter 7) and imazethapyr (Chapter 8) reduced the growth of chickpea plants. Shoot biomass was reduced by chlorsulfuron, but not by imazethapyr. The addition of nitrogen fertiliser did not alleviate the effects of either chlorsulfuron or imazethapyr, on root biomass, root length density or shoot area, suggesting that the plants are unlikely to recover after herbicide application. The addition of nitrogen fertiliser did not increase the plant nitrogen of chickpeas grown with either imazethapyr or chlorsulfuron in the soil. The fact that plant nitrogen of herbicide treated plants did not reach the same level as those grown without herbicide following the addition of nitrogen fertiliser, may be a reflection of the reduction in root biomass and root length density with reduced opportunity for nitrogen uptake. The reduction in root biomass and growth rates is due to the mode of action of the herbicide and was discussed in Chapter 7.

The plants that were inoculated with rhizobia pre-exposed to either chlorsulfuron or imazethapyr, particularly those pre-exposed to chlorsulfuron, had a reduced root biomass and responded to nitrogen fertiliser through an increase in biomass. The greater response to nitrogen fertiliser may indicate ineffective symbiosis. It is possible that the nitrogen requirements of the plants inoculated with pre-exposed rhizobia (either chlorsulfuron or imazethapyr) were not met by nitrogen fixation alone and therefore the plants responded more to the additional available nitrogen. However, the plant nitrogen results from the imazethapyr experiment showed that there was little difference in nitrogen content between plants inoculated with either pre-exposed or non pre-exposed rhizobia. The data also showed however, that there was an interaction between rhizobia and soil treatments, and were averaged over both + and – nitrogen data. Therefore, conclusions as to whether the uptake of additional nitrogen actually increased plant biomass cannot be drawn.

8.4.4 Comparison of the effects of chlorsulfuron (Chapter 7) and imazethapyr (Chapter 8) on nodulation and nitrogen of chickpeas grown in pots, under controlled conditions.

Although experimental conditions were identical, the number and weight of nodules in the imazethapyr experiment were lower than in the chlorsulfuron experiment, but as the results of each experiment were discussed relative to their respective controls, and the overall trends observed were similar, they will be discussed together. Imazethapyr, like chlorsulfuron, reduced the number of nodules on chickpea roots. The greatest reductions in nodule number were observed when the herbicides (chlorsulfuron or imazethapyr) were present in the soil in which the plants were grown. The reduction in nodulation may be a reflection of the reduction in root biomass, following herbicide

application, leading to fewer root hairs and therefore fewer available infection sites (Martensson and Nilsson, 1989).

The number of nodules was also reduced in chickpea plants inoculated with rhizobia pre-exposed to either chlorsulfuron or imazethapyr. This was observed even when these herbicides were not present either in the soil or during germination. This reduction in nodulation due to pre-exposure of nodules has not been observed with ALS-inhibiting or other herbicides, prior to the studies in Chapters 7 and 8, and may be unique to chickpeas. The consequences and possible mechanisms for this reduction in nodulation due to pre-exposure of rhizobia to chlorsulfuron or imazethapyr were discussed in Chapter 7 and will be discussed further in Chapters 9 and 10.

Unlike chlorsulfuron, pre-exposure of rhizobia to imazethapyr did not reduce nodule weight. The reduction in nodule weight due to pre-exposure of rhizobia to chlorsulfuron is probably a reflection on the reduced number of nodules. However, imazethapyr reduced number but not weight of nodules, suggesting that the herbicide is reducing the number of infections rather than the size of the nodules.

When inoculated chickpeas were grown in the absence of nitrogen, both imazethapyr and chlorsulfuron reduced the plant nitrogen inferring that both herbicides reduced the amount of nitrogen fixed by chickpeas. As with chlorsulfuron, the addition of nitrogen fertiliser in the imazethapyr experiment doubled the slope of the nodule weight and plant nitrogen regression compared to those plants grown without nitrogen. The slopes of the plant nitrogen on nodule weight regression for plants inoculated with rhizobia pre-exposed to chlorsulfuron was higher than that for non pre-exposed rhizobia. However, the slopes of the plant nitrogen on nodule weight regression for

plants inoculated with rhizobia pre-exposed and not pre-exposed to imazethapyr were both similar. This difference between the two herbicides may be because chlorsulfuron had a greater impact on the nodulation of chickpea plants, reducing both number and weight of nodules and, therefore, the plant may be attempting to compensate in some way for the reduction in nodulation. However, the slopes of the plant nitrogen on nodule weight regression of plants grown in soil with and without imazethapyr or chlorsulfuron, were similar. The similarity of slopes suggests that the presence of either herbicide in the growth medium did not affect the amount of nitrogen produced per unit of nodule weight. Each unit of nodule weight is capable of fixing the same amount of nitrogen in the presence and absence of the herbicides (chlorsulfuron or imazethapyr). Therefore, the reduction in nitrogen content of chickpea plants due to the presence of herbicide is because of fewer nodules to fix nitrogen, rather than on the nodules' ability to fix nitrogen.

8.4.5 Implication of results

Including legumes in cropping rotations can provide benefits to farming systems (Unkovich *et al.*, 1997; Peoples *et al.*, 1995b; Peoples *et al.*, 1992; Angus, 1992; Sprent and Sprent, 1990). Cereal crop yields following crop or pasture legumes are usually at least 30 – 50% higher than those derived from continuous cropping with cereals (Unkovich *et al.*, 1997; Evans *et al.*, 1991). This increase in cereal yield can probably be attributed to a combination of increased nitrogen availability from legumes, reduced weed competition and breaking of disease cycles (Unkovich *et al.*, 1997; Peoples *et al.*, 1995b). The combination of conserved soil nitrogen, greater mineralisation potential, and return of fixed nitrogen in vegetative residues benefit crops following legumes (Peoples *et al.*, 1995b; Ladd, 1992; Peoples and Craswell, 1992). Both crop and pasture

legumes have been found to increase plant-available nitrate nitrogen in the soil through reduced nitrate usage, release of products of nitrogen fixation from nodulated roots, or from nitrogen mineralised from fallen leaves or roots and nodules lost during growth and development (Peoples *et al.*, 1995b). However, if the use of the herbicides studied in Chapters 7 and 8 resulted in reduced nitrogen fixation, the benefit of using legumes in a cropping rotation would be diminished. The ALS-inhibiting herbicides provide a relatively easy and economical means of controlling weeds in the farming system. However, these studies have shown that their use may cost the farmer as a result of chlorsulfuron residues remaining from use in a previous cereal crop, or through in-crop use of imazethapyr in a chickpea crop. The results from Chapters 7 and 8 suggest that plant biomass production, nodulation and nitrogen fixation are affected by both of these herbicides. Chlorsulfuron and imazethapyr severely reduced the root biomass of chickpeas. This reduction will result in less below-ground nitrogen left for future crops. The fact that the addition of nitrogen did not improve the biomass of plant roots grown in the presence of chlorsulfuron residues or imazethapyr suggests that the plants will not recover, leading to possible loss of yield and nitrogen residues. It is believed that nitrogen from the turnover of roots and nodules of legumes could provide mineralisable nitrogen for subsequent cereal crops, but little work has been undertaken to determine the importance of this below ground biomass (Unkovich *et al.*, 1997; Peoples *et al.*, 1995b). The root biomass of lupins (average available data) contained 22 kg N ha^{-1} at peak biomass and declined to 14 kg N ha^{-1} at maturity. It is assumed that the reduction in nitrogen at maturity is due to returns to the soil of roots and nodules (Unkovich *et al.*, 1997).

The results from both the chlorsulfuron and imazethapyr experiments showed that nodulation was affected by these herbicides. If nodulation is reduced, there is a chance that nitrogen fixation is also reduced. If a reduction in fixed nitrogen due to either imazethapyr or residues of chlorsulfuron was observed in the field, it may have consequences for farmers, such as less mineral nitrogen reserves for the following crop. This represents an inefficient use of a legume in a cropping sequence and results in a cost to the farmer of reduced nitrogen for future crops and a reduction in nitrogen rich residues. Each unit of nodule mass was still able to fix the same amount of nitrogen. Pre-exposure of rhizobia to chlorsulfuron or imazethapyr led to reduced nodule weights and numbers, and again this has the possible consequence of reduced nitrogen fixation. The pre-exposure of rhizobia to chlorsulfuron, but not imazethapyr, reduced plant nitrogen, suggesting that chlorsulfuron impacts on the nitrogen status of the plant.

Overall, it appears that imazethapyr and residues of chlorsulfuron will affect the nitrogen dynamics of the farming system. Along with chlorsulfuron and imazethapyr usage, come possible consequences, including possible reduction in nitrogen fixation resulting in the legume utilising soil mineral nitrogen reserves. The reduction in root biomass and nodules due to herbicide usage, also leads to a reduction of nitrogen rich vegetative residues into the soil and therefore, less available soil nitrogen for following crops. The use of these herbicides could cost the farmer in terms of possible reductions in yield, exploitation of soil nitrogen reserves, lower levels of nitrogen rich root and shoot biomass to return to the soil, and represents an inefficient use of a legume in the cropping sequence (Peoples *et al.*, 1989).

8.4.6 Summary of Chapters 7 and 8

The results show that residues of chlorsulfuron and imazethapyr reduced biomass, shoot area, root density, nitrogen uptake and nodulation of chickpeas. The greatest impact of the herbicides was found when they were present in the soil or growth media. When herbicides were present in the soil, roots that were exposed to them were immediately affected by the cessation of cell division and growth, and consequently nutrient uptake was reduced and normal partitioning of resources was disrupted.

The results also showed that pre-exposure of rhizobia to chlorsulfuron and imazethapyr reduced the number of nodules formed on chickpea plants. This result has not previously been reported for ALS-inhibiting herbicides. Chapter 9 will investigate two possible mechanisms responsible for the reduction in nodulation due to pre-exposure of rhizobia to the herbicides. These possible mechanisms are: (i) a direct effect on the growth of rhizobia, and (ii) herbicide carryover on rhizobial cells, even after rinsing, to the site of infection.

CHAPTER 9

POSSIBLE MECHANISMS RESPONSIBLE FOR THE REDUCTION IN THE NUMBER OF NODULES ON CHICKPEA PLANTS FOLLOWING PRE-EXPOSURE OF CHICKPEA RHIZOBIA (CC1192) TO ALS-INHIBITING HERBICIDES

9.1 INTRODUCTION

The previous chapters showed that nodulation of chickpea plants was reduced by chlorsulfuron and imazethapyr. Exposing rhizobia to either chlorsulfuron or imazethapyr prior to inoculation led to a reduction in the number of nodules formed on chickpea roots. The reduction in nodulation, due to the pre-exposure of rhizobia to either herbicide, occurred in the absence of the herbicides at germination or in the soil. Reduction in nodulation, from pre-exposure, may have been due to: (i) the herbicide affecting the growth or survival of rhizobia; (ii) direct herbicide effects on roots due to some herbicide remaining on rhizobia cells after rinsing with Ringer's solution, or; (iii) exposing rhizobia to the herbicide influencing the process of root infection or nodule formation.

Studies examining the influence of ALS-inhibiting herbicides on the growth or survival of rhizobia have produced varied results. Growth of *Rhizobium meliloti* strain 14 and *Rhizobium leguminosarum* bv *trifolii* grown in pure culture were unaffected by chlorsulfuron at application rates corresponding to 50 and 500 times those recommended for the field in Sweden (4 g ha^{-1}) (Martensson and Nilsson, 1989). The

recommended rate of application in South Australia is 20 g ha^{-1}. However, in another study by Eberbach and Douglas (1989), the growth rates of *Rhizobium trifolii* were relatively normal at low levels of chlorsulfuron (0.2, 0.5 and 1.0 mg ai L^{-1} nutrient solution) but growth was inhibited when the herbicide concentration was increased to 2 mg ai L^{-1}. Martensson (1992), observed that different strains of *Rhizobium leguminosarum* bv *trifolii*, *R. Meliloti* and *R. loti* varied in sensitivity to chlorsulfuron with some tolerant and some sensitive. Levels of imazethapyr up to 7000 times the recommended application rate in Spain were required to cause slight effects on *Rhizobium* growth in a defined medium, suggesting that imazethapyr would have little effect on the growth of *Rhizobium* in the field under normal application rates (Gonzalez *et al.*, 1996). The results from these studies suggest that ALS-inhibiting herbicides may affect the growth of some rhizobia species, although the rates used in experiments are often much higher than those in the field. To date, however, no studies have been conducted to investigate the effect of these herbicides on nodulation and nitrogen fixation by chickpea *Rhizobium*.

This chapter discusses two sets of experiments designed to investigate possible mechanisms responsible for the reductions in chickpea nodulation from pre-exposure of rhizobia to ALS-inhibiting herbicides, observed in Chapters 7 and 8. The first set of experiments investigates the direct effects of the ALS-inhibiting herbicides chlorsulfuron, imazethapyr and flumetsulam on the growth of chickpea rhizobia (CC1192). The second experiment investigates whether herbicide was retained on the rhizobial cells that were pre-exposed to chlorsulfuron. There is potential for carryover of herbicide on rhizobial cells and this requires investigation. Although the quantity of

herbicide bound to rhizobial cells is likely to be small, it could still cause some effects at the target site in actively dividing root cells.

The aims of these experiments were to:

1) Study the effects of chlorsulfuron, imazethapyr and flumetsulam at double the recommended application rate on the growth of chickpea *Rhizobium*;

2) Study the effects of chlorsulfuron, in a defined media, on the growth of chickpea *Rhizobium;*

3) Investigate whether all herbicide could be removed from rhizobial cells by rinsing with ¼ strength Ringer's solution.

9.2 EFFECTS OF ALS-INHIBITING HERBICIDES ON THE GROWTH OF CHICKPEA RHIZOBIA

9.2.1 Introduction

The reduction in nodulation due to pre-exposure of rhizobia may be due to direct effects of the herbicide on growth or survival of rhizobia. The objective of this experiment was to determine the effects of chlorsulfuron, imazethapyr and flumetsulam at double the recommended application rate on the growth of chickpea *Rhizobium* at pH 7.0 and 8.0.

9.2.2 Materials and Methods

The effects of chlorsulfuron, imazethapyr and flumetsulam on the growth of chickpea rhizobia were investigated in separate experiments. For each herbicide the methods used to assess the direct effect of the herbicides were identical except for the

amount of herbicide used. Therefore, the experiments for each herbicide will be discussed together.

9.2.2.1 Treatments and experimental setup

A starter culture of chickpea rhizobia (CC1192) was prepared by growing rhizobia in yeast mannitol broth (YMB described in Chapter 6; section 6.2) on a shake incubator at 25°C for 28 hours or until an absorbance at 500 nm of approximately 1.0 (1 x 10^9 cell forming units ml^{-1}) was reached. This starter culture was used to inoculate the sample flasks in the experiments. The sample flasks were prepared by adding 20 ml of YMB to 50 ml conical flasks. The flasks were stoppered with cotton wool plugs and an aluminium foil cap was placed over each plug. The flasks were then autoclaved at 121°C for 20 minutes. The pH of YMB was initially 7.0. The experiments using chlorsulfuron and imazethapyr were first performed at pH 7.0 and then repeated at pH 8.0 to more closely resemble the field conditions at which effects of the herbicide on chickpea growth and nitrogen fixation were observed. The flumetsulam experiment was only undertaken at pH 7.0 as results from Chapters 4 and 5 found that this herbicide had no effect on plant growth or nitrogen fixation.

Solutions of the herbicides were filter sterilised (through a 0.2µm Supor® membrane filter) and added to the appropriate autoclaved flasks. The control flasks had sterile deionised water added, and Table 9.1 shows the amount of each herbicide added for each experiment. The amount of herbicide added to each flask was double the recommended field application rate (the field application was assumed to penetrate to a depth of 15 cm when the field application rate in g ha^{-1} was converted into to µg ml^{-1} in

a shake flask). This level of exposure of rhizobia to the herbicide was chosen because, if an effect was not observed at this level, then the recommended application rate would be of little or no consequence to chickpea rhizobia in the field.

Table 9.1: Application rates of the herbicides used in experiments investigating the effects of ALS-inhibiting herbicides on the growth of chickpea rhizobia.

Herbicide	Application rate
Chlorsulfuron	0.02 μg ai ml^{-1}
Imazethapyr	0.04 μg ai ml^{-1}
Flumetsulam	0.027 μg ai ml^{-1}

After either herbicide or water had been added to the flasks they were inoculated with 1 ml of the rhizobia starter culture. When measuring absorbance, control flasks (zero absorbance) were left uninoculated. All flasks were then placed on a shake incubator at 25°C for up to one week.

9.2.2.2 Experimental design

Table 9.2 summarises the treatments for the experiments discussed in this section. The flasks were set up with and without herbicide. The flasks without herbicide will be referred to as 'controls' in the results. Flasks were removed from the experiment at each sampling time, either every four or eight hours (Table 9.2). The treatments were duplicated at each sampling time. At each sampling an uninoculated flask containing YMB was removed and used to zero the spectrophotometer. The differences in growth rates for each herbicide were detected using an exponential model, which is one form of non-linear regression. The need for separate non-linear

and linear parameters for each group was tested using an F-test. All analyses were performed in Genstat Release 4.1 (Payne, 1993).

Table 9.2: Summary of treatments and experiments investigating the effects of flumetsulam, chlorsulfuron and imazethapyr on the growth of chickpea rhizobia. +/- indicates presence or absence of the herbicide.

Herbicide	pH	Treatment		Sampling interval (hours)
Flumetsulam	7.0	+	-	4
Chlorsulfuron	7.0	+	-	8
Imazethapyr	7.0	+	-	8
Chlorsulfuron	8.0	+	-	8
Imazethapyr	8.0	+	-	8

9.2.2.3 Sampling and measurements

In the flumetsulam experiment, samples were taken for optical density every four hours for 48 hours and then at 72, 120 and 168 hours. As the results from the flumetsulam experiment found no difference between control and herbicide treatments, it was decided that sampling could be undertaken every eight hours rather than every four. Consequently this time interval (8 hours) was also used in the chlorsulfuron and imazethapyr experiments. Optical density was measured using a GBCUV/VIS 916 spectrophotometer connected to a personal computer running the GBCUV general methods program.

Plate counts were taken every eight hours for the control treatments using the drop plate method with an eight fold dilution series and phosphate buffer as the base. The plates were incubated at 25°C until distinct, 'countable' colonies formed. These were counted and the number of colony forming units per ml, from the original flask,

calculated. Plate counts from the control group were plotted against optical density and this was used to estimate the number of colony forming units for each herbicide treatment.

9.2.3 Results

Rhizobia followed the classic bacterial growth pattern (Drew, 1981) with an initial lag phase, a rapid growth (exponential) phase and a stationary phase (Figure 9.1). There was no difference in optical density between the chlorsulfuron and the control treatment at pH 7.0 (Figure 9.1). This trend was also observed with flumetsulam and imazethapyr. In addition, increasing the pH of the culture solution for chlorsulfuron or imazethapyr from 7.0 to 8.0 did not alter the results.

Figure 9.2 shows the number of rhizobia colonies formed on agar plates plotted against optical density for the control or no herbicide treatments at pH 7.0. This allowed the colony forming units to be calculated for each herbicide from optical density measurements.

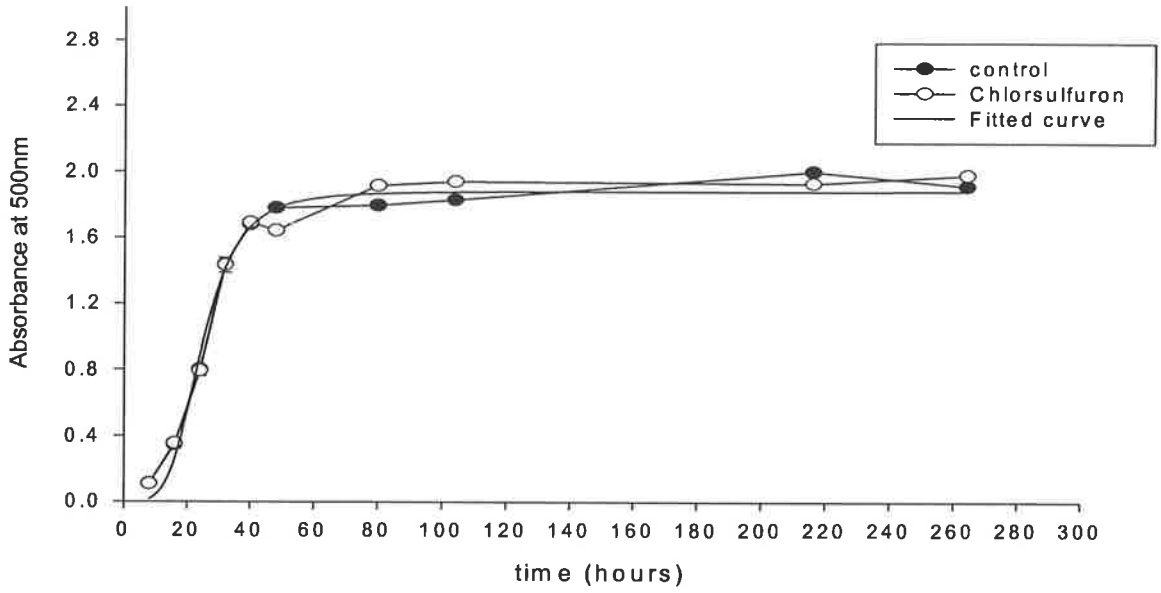


Figure 9.1 The effects of chlorsulfuron on chickpea rhizobia growth at pH 7.0 as measured by optical density (bars indicate standard error of mean which were very small). The data were fitted to an asymmetric sigmoidal model: y= min+(max-min)/(1+X/X50)$^{-p}$) where y equals absorbance at 500 nm, X equals time (h), X50 equals μmax (point of maximum specific growth rate (Robinson & Tiedje, 1983)), p equals slope parameter, max and min are the maximum and minimum absorbances of the culture respectively (r^2 = 0.99).

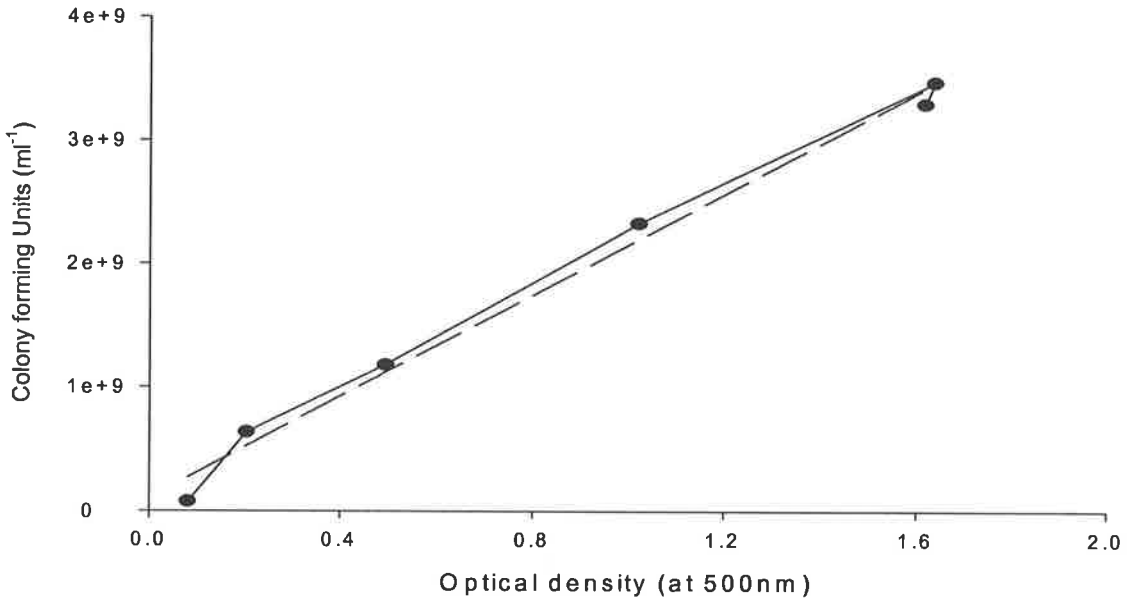


Figure 9.2 The number of chickpea rhizobia colony forming units as predicted by optical density. Dashed line is the line of best fit. (cfu=1.06 x 10^8 + 2.05 x 10^9 OD. Where cfu = colony forming units and OD = optical density; r^2 = 0.99).

Doubling time (the time taken optical density to double) and μmax (maximum specific growth rate) values for all herbicides at pH 7.0 and 8.0 are shown in Table 9.3. There was no significant difference in the doubling times between control and herbicide treatments at either pH 7.0 or 8.0 (Table 9.3). The μmax were between 24 and 30 hours for all herbicides at pH 7.0, but took longer to reach at pH 8.0 with μmax values of 39 and 38 hours for chlorsulfuron and imazethapyr respectively (Table 9.3).

Table 7.3: Doubling times and maximum specific growth rates (μmax) of chickpea rhizobia grown in solution culture in the presence of flumetsulam, chlorsulfuron and imazethapyr at pH 7.0 and 8.0. μmax and r^2 values were obtained from the model described at Figure 9.1.

Herbicide	Rate of application	pH	Doubling time in exponential phase (hours)	μmax	r^2
Control		7.0	8	24.63	0.99
Flumetsulam	0.027 μg ai ml^{-1}	7.0	8	28	0.99
Chlorsulfuron	0.02 μg ai ml^{-1}	7.0	8.5	25.6	0.99
Imazethapyr	0.04 μg ai ml^{-1}	7.0	8.5	29.5	0.99
Control		8.0	8.5	38	0.99
Chlorsulfuron	0.02 μg ai ml^{-1}	8.0	8	39	0.99
Imazethapyr	0.04 μg ai ml^{-1}	8.0	8	38	0.99

9.3 EFFECTS OF CHLORSULFURON ON CHICKPEA RHIZOBIA GROWN IN A DEFINED MEDIA

9.3.1 Introduction

In the previous growth experiments, rhizobia were grown in yeast mannitol broth containing amino acids. As ALS-inhibiting herbicides act by reducing the production of branched chain amino acids, it was possible that the presence of branched chain amino acids in the YMB may have masked the effects of herbicide on rhizobia growth. The objective of this experiment was to determine the effects of the herbicide chlorsulfuron on the growth of chickpea rhizobia in the absence of amino acids (defined media).

9.3.2 Materials and Methods

9.3.2.1 Treatments and experimental setup

Rhizobia were grown in a defined media (Table 9.4), containing no amino acids. The pH of the media was adjusted to 8.0 using NaOH. The experiment was set up as a dose response curve with four rates of chlorsulfuron addition (Table 9.5).

Table 9.4: Defined growth media used to investigate the effects of chlorsulfuron on chickpea rhizobia growth (from Brown and Dilworth, 1975).

Chemical	Mass (mg L^{-1})
Glucose	2500
KH_2PO_4	360
K_2HPO_4	1400
$MgSO_4.7H_2O$	250
$CaCl_2.2H_2O$	20
NaCl	200
$FeCl_3$	6.6
EDTA	15
$ZnSO_4.7H_2O$	0.16
$NaMoO_4$	0.2
H_3BO_3	0.25
$MnSO_4.4H_2O$	0.2
$CuSO_4.5H_2O$	0.02
$CoCl_2.6H_2O$	0.001
Thiamine –HCl	1
Calcium pantothenate	2
Biotin	0.001
KNO_3	700

Table 9.5: Application rates of chlorsulfuron used in the experiment investigating the effects of the herbicide on the growth of chickpea rhizobia grown in a defined media.

% of recommended application rate	Amount of chlorsulfuron added
0	0 (control)
50 (1/2 recommended application rate)	5×10^{-3} µg ai ml^{-1}
100 (recommended application rate)	0.01 µg ai ml^{-1}
200 (double recommended application rate)	0.02 µg ai ml^{-1}

9.3.2.2 Experimental design and sampling

The four treatments in the experiment were nil, half, recommended and double the recommended field application rate (Table 9.5) and each treatment was duplicated. Zero absorbances were obtained by including 9 additional flasks that were not

inoculated. The whole experiment consisted of a total of 81 flasks. Optical density (at 500 nm) of samples taken from the flasks was measured every 8 hours for 48 hours and then again at 72 hours as described in section 9.2.2 above. Significant differences were determined by non-linear regression as in section 9.2.2 above.

9.3.3 Results

The rate of chlorsulfuron addition did not significantly alter the growth rate of chickpea rhizobia (p=0.270) (Figure 9.3).

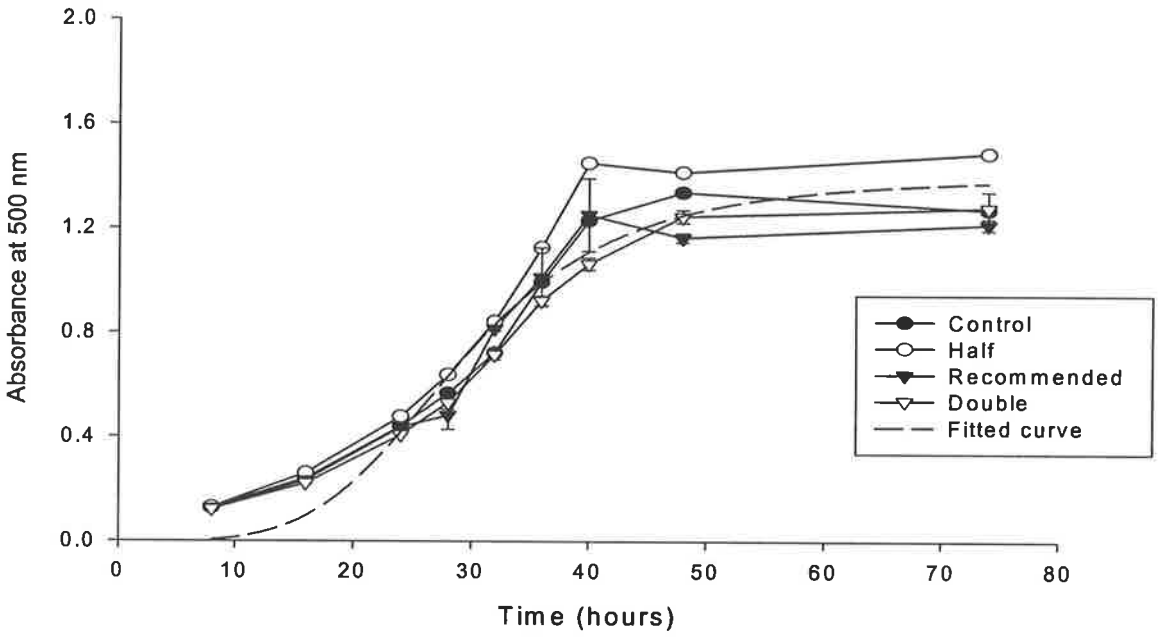


Figure 9.3: Dose response curve for chickpea rhizobia affected by chlorsulfuron (bars indicate standard error). The data were fitted to an asymmetric sigmoidal model: $y= min+(max-min)/(1+X/X50)^{-p}$) where y equals absorbance at 500 nm, X equals time (h), X50 equals μmax (point of maximum specific growth rate (Robinson & Tiedje, 1983)), p equals slope parameter, max and min are the maximum and minimum absorbances of the culture respectively. ($r^2 = 0.99$).

9.4 DOES RINSING WITH ¼ RINGER'S SOLUTION REMOVE ALL THE HERBICIDE FROM RHIZOBIAL CELLS?

9.4.1 Introduction

It is possible that the reduction in nodulation measured in the experiments in Chapters 7 and 8 in response to pre-exposure of the rhizobia to herbicide, was caused by residual herbicide bound to rhizobial cells. If the herbicide was not removed from the rhizobial cells, it would be taken directly to the dividing root cells, the site of action of the herbicide. This experiment was designed to determine whether the rinsing protocol used in the experiments described in Chapters 7 and 8 was effective in removing all of the herbicide. The objective of this experiment was to determine if any herbicide remained adsorbed to the rhizobial cells after rinsing with ¼ strength Ringer's solution using ^{14}C labelled chlorsulfuron.

9.4.2 Materials and methods

Two treatments, consisting of either ^{14}C labelled chlorsulfuron or unlabelled chlorsulfuron, were used in the experiment. Flasks (250 ml conical flasks) were set up as a static culture (Plate 9.1). Rubber bungs were placed in each flask and a 20 cm needle was placed through each to carry filter sterilised air into the flask (Plate 9.1). The flasks were sealed by taping around the bungs to aid in sterility and to prevent the bungs from popping out during autoclaving (Plate 9.1). Each flask contained 100 ml of yeast mannitol broth (Plate 9.1). The flasks were autoclaved at 121°C for 20 minutes with the bungs and needles, along with all tubing required to carry air into and out of each flask and the 0.2 μm (Millex® - FG) filter to be used for the air.

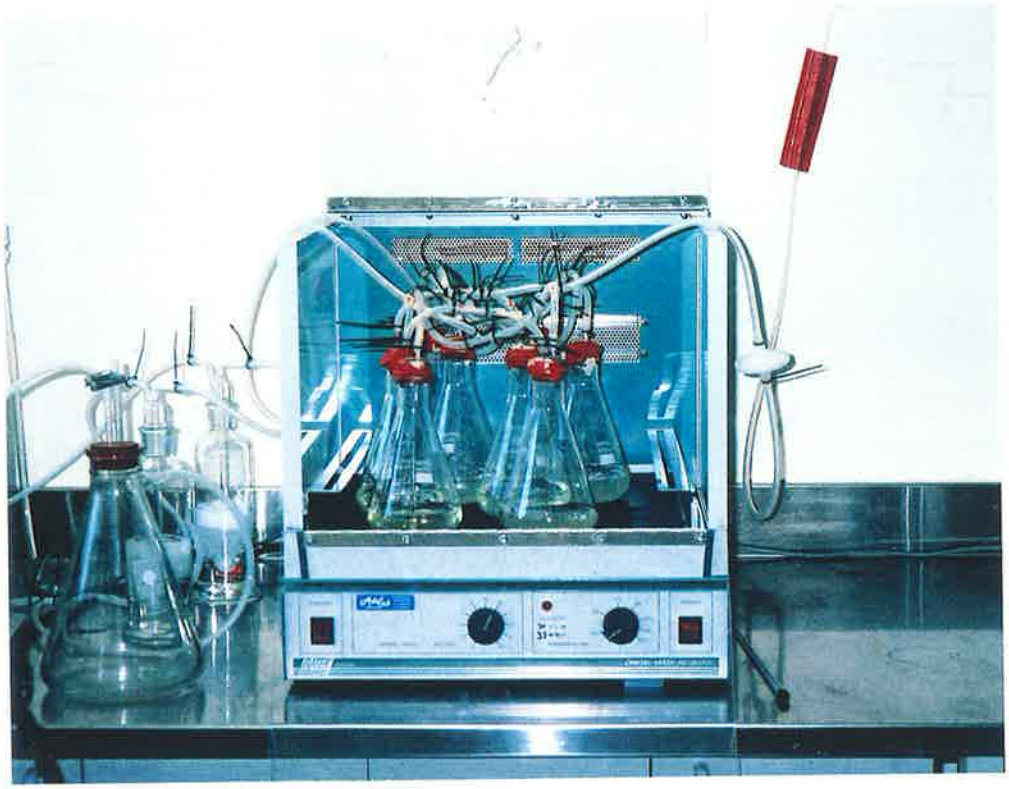


Plate 9.1: Experimental set up of an experiment to investigate whether rinsing with 1/4 strength Ringer's solution removed all ^{14}C labelled chlorsulfuron from rhizobial cells. There were four replicates of two treatments (labelled and unlabelled herbicide). Rhizobial cells were grown in yeast mannitol broth. The flasks were stoppered with a rubber bung and a 20 cm needle carried filter sterilised air into the flasks. Outlet tubes were connected to NaOH traps to collect CO_2 given off during the experiment.

After autoclaving, the flasks were allowed to cool, and a filter sterilised herbicide solution (either labelled or unlabelled) was added (2.5 ml of a 1 ppm solution). The ^{14}C labelled 1 ppm solution consisted of 57 μg labelled and 43 μg unlabelled chlorsulfuron that equated to 1.12×10^7 Bq or 1.86×10^5 dpm (disintegrations per minute). The flasks were then inoculated with rhizobia and set up in an incubator at 25°C. The outlet tubes for each flask were connected to a 5 N NaOH trap to collect any CO_2 given off during the experiment (Plate 9.1). There were two NaOH traps – one for each of the labelled and unlabelled herbicides (Plate 9.1). Air was then bubbled through the flasks for 48 hours. There were four flasks for each treatment and these were bulked for analysis so that enough labelled material could be collected to allow for effective ^{14}C measurement (Plate 9.1).

After 48 hours, the rhizobia cultures were decanted into sterile centrifuge tubes and centrifuged for 10 minutes at 15,000 rpm using a Sorvall Superspeed RC 2-B centrifuge. After this the flasks were again decanted, the supernatant of the labelled herbicides was kept aside for ^{14}C activity measurement, and the remaining rhizobial cells were resuspended in ¼ strength Ringer's solution and shaken. The four flasks from each treatment were then combined, leaving only one centrifuge tube for each treatment. The flasks in which labelled herbicide had been used were rinsed with methanol to remove any herbicide remaining on the flask walls. These were also combined and a sample retained in order to later measure the ^{14}C activity. The cells were then re-centrifuged, the supernatant decanted and retained and the cells were once again resuspended. This process was repeated another 2 times with each resulting supernatant kept after centrifuging.

The ^{14}C activity associated with each supernatant, the final rinsed cells of both the labelled and unlabelled treatments, the NaOH trap, and the rinsed flasks was measured. Table 9.6 lists samples taken for ^{14}C activity analysis and Figure 9.4 presents a flow chart outlining sample collection. Drop plate counts were taken from the final rinsed cells in order to determine the number of colonies formed.

The activity of the samples was measured using a Win Spectral liquid scintillation counter. Nine ml of scintillant were added to 1 ml of sample in 10 ml glass scintillation vials. The vials were shaken and then placed into racks in the Win Spectral machine. Activity was measured and a mass balance calculation was completed for ^{14}C to ensure that all labelled herbicide was accounted for. Expected equivalent levels of chlorsulfuron in the field were calculated using the results from the mass balance.

Table 9.6: Description of samples taken to determine mass balance following ^{14}C chlorsulfuron addition during rhizobial growth.

Sample name	Description
Supernatant 1	Collected after first centrifuge, prior to any rinsing
Supernatant 2	Collected after first rinse
Supernatant 3	Collected after second rinse
Supernatant 4	Collected after third and final rinse
Rinsed Cells	Rhizobial cells after third rinse and after decanting supernatant
Rinsed flasks	Conical flasks were rinsed with methanol to remove any herbicide adhering to walls
NaOH trap	Set up to collect any labelled CO_2 given off by rhizobia
Control cells	Rhizobial cells from unlabelled herbicide treatment following 3 rinses.

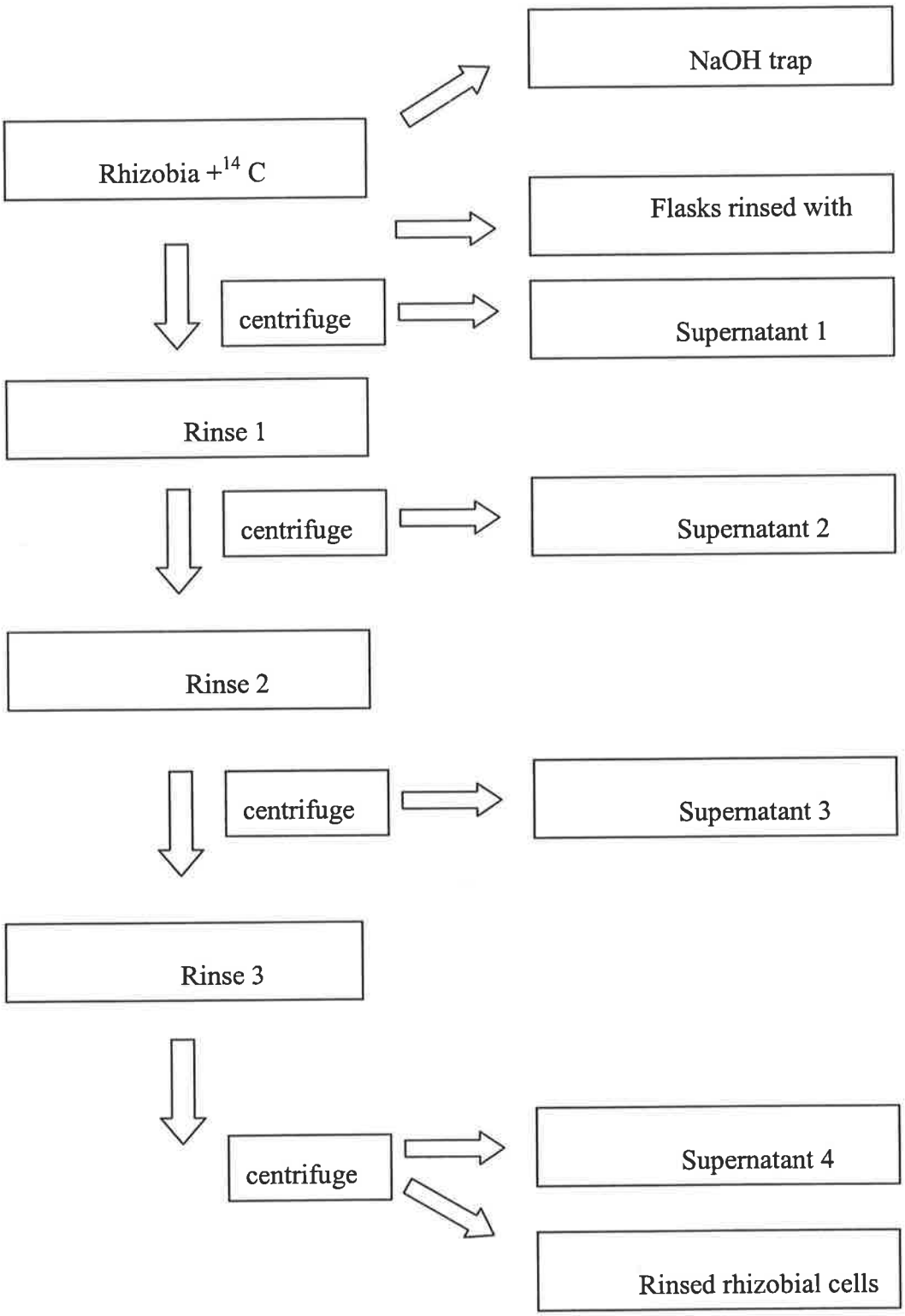


Figure 9.4: Flow chart outlining the collection of samples to determine the activity of ^{14}C chlorsulfuron remaining at each stage.

9.4.3 Results

Results of the mass balance are presented in Table 9.7. Herbicidal activity measured at each stage of the rinsing process is presented as a percentage of the original amount of herbicide added. All herbicide was accounted for (Table 9.7) at various stages of rinsing. The majority of the labelled herbicide (92%) was removed at the time of the first centrifugation, prior to rinsing (Table 9.7). The first rinse removed a further 5% of the herbicide and approximately 1% was left attached to the rhizobial cells following the third and final rinse (Table 9.7). The plate counts found 7 x 10^9 rhizobia colony forming units per ml were formed following the rinsing process.

Table 9.7: Mass balance of ^{14}C labelled chlorsulfuron experiment investigating whether rinsing with saline solution removed all of the herbicide from the rhizobial cells.

Stage of rinsing	% of original
Supernatant 1	92.09
Supernatant 2	5.45
Supernatant 3	0
Supernatant 4	0
Rinsed flasks	2.06
Rinsed cells	1.20
NaOH trap	0
Control	0
Total	100.8

9.5 DISCUSSION

The herbicides chlorsulfuron, flumetsulam and imazethapyr, did not influence the growth of chickpea rhizobia, at either pH 7.0 or 8.0, in yeast mannitol broth or in the absence of amino acids in the defined media. The results clearly showed that the herbicides evaluated did not affect the growth or doubling time of the rhizobia under the conditions of the experiments. Therefore, the reduction in nodulation observed in Chapters 7 and 8, cannot be explained by a direct effect of the herbicide on the growth of rhizobia. This is consistent with published literature where ALS-inhibiting herbicides had no effect on the growth of rhizobia, except at unrealistic levels and were discussed in section 9.1 (Gonzalez *et al.*, 1996; Martensson and Nilsson, 1989).

A follow-up study was undertaken to determine if any herbicide was adsorbed to the rhizobial cells after rhizobia were pre-exposed to chlorsulfuron and rinsed with Ringer's solution. Using ^{14}C labelled chlorsulfuron only 1% of the added chlorsulfuron was adsorbed to and not rinsed off rhizobial cells. The 1% adsorption from an initial 1ppm stock solution (consisting of 57 µg labelled and 43 µg unlabelled herbicide) equates to 0.0143 µg ml^{-1} on the rinsed rhizobial cells used to inoculate each pot in the nodulation experiment discussed in Chapter 6 (Table 9.8). This adsorption equates to approximately 1.43 x 10^{-11} g ai g^{-1} soil or 2.04 x 10^{-18} g ai $cell^{-1}$ for each rhizobial cell (Table 9.8). This level of 1.43 x 10^{-11} g ai g^{-1} soil is approximately three orders of magnitude (1/1000) lower than the recommended field application rate of 1.5 x 10^{-8} g ai g $soil^{-1}$ assuming uniform distribution in the top 15 cm of the soil (Table 9.8). Gillett and Holloway (1996), showed inhibited root growth of *Medicago truncatula* at triasulfuron levels of 2 x 10^{-9} g ai g $soil^{-1}$, and chlorsulfuron inhibited seedling growth

of *M. sativa* and *M. scutellata* grown in a soil free system at levels of 2×10^{-10} g ai L^{-1} (Jettner *et al.*, 1999). The growth of lentils was also inhibited by chlorsulfuron at levels of 2×10^{-10} g ai g $soil^{-1}$ in field trials in Canada (Moyer *et al.*, 1989). These concentrations of chlorsulfuron observed by Jettner *et al.* (1999) and Moyer *et al.* (1989) are the lowest concentrations found to inhibit growth of legumes reported in the literature to date. The results from these studies, in addition to the nodulation studies discussed in Chapters 7 and 8, suggest that the level of chlorsulfuron remaining on rhizobial cells after rinsing, would be inadequate to inhibit the growth of chickpea roots and nodulation in chickpeas. The level of chlorsulfuron found on chickpea rhizobia after rinsing would probably have little impact on the roots themselves. Therefore, it is unlikely that the reduction in nodulation noted in Chapter 7, when rhizobia were pre-exposed to chlorsulfuron, resulted from an influence of the chlorsulfuron on root biomass and therefore the number of potential sites of infection.

Table 9.8: Chlorsulfuron carryover on rhizobial cells and how this equates to conditions in the pot and in the field.

	Chlorsulfuron
Rinsed cells	0.0143 μg ml^{-1}
Inoculant (1ml pot^{-1})	1.43×10^{-8} g ai pot^{-1}
Soil (1 kg pot^{-1})	1.43×10^{-11} g ai g $soil^{-1}$
Rhizobial cells	7×10^{9} cells ml^{-1}
	2.04×10^{-18} g ai $cell^{-1}$
Field (recommended application rate when evenly distributed in the top 15 cm of soil)	1.5×10^{-8} g ai g $soil^{-1}$

Rhizobia multiply in the rhizosphere in response to secretions of plant exudates that contain organic compounds (Bowen and Rovira, 1976; Nutman, 1975).

Furthermore, not all rhizobia present in the soil will infect the plant root hair by means of infection threads and the majority will remain on the root surface or in the mucigel layer of the root (Rolfe and Richardson, 1987; Nutman *et al.*, 1978). This suggests that the probability of the rinsed rhizobia entering the infection thread and carrying enough chlorsulfuron to the herbicide site of action in the meristem, is very low. It appears likely that another factor may be responsible for the effect of pre-exposure of rhizobia on nodulation in chickpea.

The studies reported in this chapter have established which mechanisms are not responsible for herbicide inhibition of nodulation in chickpeas:

i) no direct effect of herbicides on rhizobia growth;

ii) herbicide carryover on rhizobia to affect nodulation.

It is possible that chlorsulfuron is interfering with the nodule infection process. This is a complex process involving an exchange of molecular signals between the bacterium and the host plant (Hungria and Stacey, 1997). This will be discussed further in Chapter 10, the general discussion.

CHAPTER 10

GENERAL DISCUSSION

10.1 DISCUSSION OF RESULTS

ALS-inhibiting herbicides provide an economical and efficient means of controlling weeds in farming systems (Brown, 1990; Beyer *et al.*, 1987). However, there is evidence to suggest their use may negatively impact on legume crops and pastures in farming rotations (Table 2.6). This study set out to determine the effects of selected sulfonylurea residues and/or in-crop use of flumetsulam and imazethapyr on: (i) the growth and yield of medic; (ii) the growth, yield and symbiotic nitrogen fixation of chickpea; and (iii) possible mechanisms for any effects observed. The study concentrated on the influence of ALS-inhibiting herbicides on chickpeas as little previous work had investigated the effects of these herbicides on this crop.

Application of flumetsulam at the recommended rate (20 g ai ha^{-1}) inhibited the growth of chickpea plants grown in pots in a glasshouse. However, flumetsulam only inhibited nodulation of chickpeas when high levels of nitrogen fertiliser were applied. This inhibition of nodulation was likely due more to the high inorganic nitrogen concentration in the soil, suppressing nodulation, rather than herbicide effects (Jessop *et al.*, 1984), as inorganic nitrogen also reduced nodulation in the absence of flumetsulam. The amount of nitrogen taken up by chickpea plants was also reduced by flumetsulam, but % N concentration of chickpea plants was not. The addition of nitrogen fertiliser did not alleviate the reduction suggesting that, through a reduction in chickpea root biomass, flumetsulam reduced the ability of the chickpea root system to extract nitrogen

from the soil, and/or translocate nitrogen throughout the plant. In addition, the reduction in nodulation, following flumetsulam application, would reduce the amount of plant nitrogen by limiting the fixation of atmospheric N_2. These results from pot experiments suggested that caution may be required when using flumetsulam on chickpea crops in the field. However, field trials (Chapter 4 and Chapter 5) showed that flumetsulam (applied post sowing at 20 g ai ha^{-1}) did not reduce either shoot biomass production or seed/grain yield of medic or chickpea. De Vries (1980), noted that caution should be taken when extrapolating the results of pot experiments to the field, mainly due to the spatial restriction of roots in pot experiments. In the pot experiment completed in this thesis, flumetsulam was prevented from leaching out of the soil by lining the pots with plastic bags. However, in field trials, there is potential for the herbicide to move down the soil profile and out of the zone of root activity. In addition, field and pot trials have differing root, nutrient and herbicide profiles that alter the pattern of absorption and uptake (Jettner *et al.*, 1999). The results of the field trial (Chapter 4) support the findings of other authors who demonstrated that flumetsulam gave good weed control and did not affect the yield of pasture species in South Australia (Dickinson *et al.,* 1993; Ewers and Phillips, 1993). Whilst the observations of Dickinson *et al.* (1993) support those in this study, it is not clear if pasture species and their rhizobial symbionts are equally susceptible to herbicide impacts as chickpeas. Although the results from the pot experiment showed some negative impacts of the herbicide, data from the field experiment and observations from Ewers and Phillips (1993) and Dickinson *et al.* (1993) suggest that flumetsulam is safe to use at recommended application rates in chickpea crops and medic pastures.

In the growing season following application, residues of both chlorsulfuron and triasulfuron reduced shoot biomass production of medic plants in the field (Chapter 4). This is likely a result of direct herbicide toxicity to the plant, that acted by inhibiting cell division and growth (Brown *et al.*, 1990; Beyer *et al.*, 1987; Ray, 1982a). A similar reduction in medic shoot biomass due to chlorsulfuron and triasulfuron residues was observed by Gillett and Holloway (1996) and Evans *et al.* (1993) and was discussed in Chapter 4. The reduction in shoot biomass has implications for grazing practices as less fodder for stock will be available. Therefore, caution should be taken to observe plant back periods (as a minimum) on herbicide labels for medic, following the use of chlorsulfuron or triasulfuron herbicides in cereal crops. Seed yield of *M. rugosa* was unaffected by chlorsulfuron and triasulfuron twelve months after their application. However, Evans *et al.* (1993) reported a reduction in seed yield following chlorsulfuron application. The observed differences in seed yields between the two studies, may have been due to the higher pH (and therefore slower herbicide degradation) of the field trial in the study by Evans *et al.* (1993) and was discussed in Chapter 4.

Previous investigations reported that chlorsulfuron residues of between 3% to 15% of the original application rate remained in alkaline soils (pH>8.0) 12 months after application (Black *et al.*, 1999; Walker and Robinson, 1996; Vicari *et al.*, 1994). The variation in residual levels was attributed to differences in rainfall distribution (Black *et al.*, 1999; Walker and Robinson, 1996; Vicari *et al.*, 1994). The simulated residual levels of 10 and 20% of the recommended application rate of chlorsulfuron, used in the field trial (Chapter 5), could be considered average and high respectively compared to the literature values. Simulated residual levels of chlorsulfuron (10 and 20% of the recommended application rate) and/or imazethapyr (at the recommended application

rate) reduced shoot biomass, nitrogen fixation and to a lesser extent yield of chickpeas. Yield was reduced by the combination of 20% of the recommended application rate of chlorsulfuron and in-crop use of imazethapyr. Although reductions in shoot biomass were observed at flowering, the results indicated that imazethapyr or chlorsulfuron residues alone did not reduce grain yield of chickpea, however, this was influenced by climatic conditions late in the season. Yield appeared to be much more responsive to rainfall in the post flowering period, than to the initial herbicide damage. Even though yield was not affected by the herbicides when applied alone, chlorsulfuron residues and in-crop application of imazethapyr reduced biologically fixed nitrogen. The chlorsulfuron effects on nitrogen fixation may have been due more to adverse effects on shoot biomass, than directly on fixation, as the reduction in the amount of nitrogen fixation reflected the observed reduction in shoot biomass. These reductions in nitrogen fixation, even if a reflection of biomass reduction, have implications for the nitrogen balance of the soil and therefore, availability of nitrogen for the subsequent cereal crop. The use of either chlorsulfuron or imazethapyr may reduce the nitrogen benefits normally observed subsequent to legume use in a cropping sequence. The implications of this will be discussed later in this chapter (Section 10.2).

The reduction in nitrogen fixation following chlorsulfuron or imazethapyr application may be due to: (i) a direct herbicide effect on the plant, (ii) the herbicide affecting nodulation, or (iii) the herbicide affecting rhizobia. Following the observed reduction in nitrogen fixation, further experiments were conducted in order to investigate the possible mechanisms responsible. Experiments were completed to determine the effects of chlorsulfuron and imazethapyr on nodulation of chickpea. Results showed that both chlorsulfuron and imazethapyr reduced plant biomass, shoot

area, root density, nitrogen uptake, and nodulation of chickpeas. Nodulation was reduced by the presence of imazethapyr or residual levels of chlorsulfuron in the soil, and was considered to be a result of root damage and reduced root biomass leading to fewer potential infection sites for rhizobia. When chlorsulfuron or imazethapyr were present in the soil, roots that contacted the herbicide were immediately affected by the cessation of cell division and growth (Brown, 1990; Beyer *et al.*, 1988; Ray, 1982a). Consequently nutrient uptake was potentially reduced by the reduced root biomass (Ferris *et al.*, 1992) and normal partitioning of resources was disrupted. However, the linear regressions (Chapters 7 and 8) demonstrated that there was no difference in the ability of root nodules of plants grown in soil, with or without, chlorsulfuron or imazethapyr (and in the absence of nitrogen), to fix nitrogen. It appears that the reduction in plant nitrogen and hence nitrogen fixation, of chickpeas grown in soil with chlorsulfuron residues or imazethapyr, was due to a herbicide induced reduction in the number of nodules, rather than on the ability of nodules to fix nitrogen. The observed reduction in nodule number was consistent with other studies where chlorsulfuron inhibited early root hair infection, or the number of nodules formed, of *Medicago sativa* (Koopman *et al.*, 1995; Martensson, 1992; Martensson and Nilsson, 1989), sub clover (Eberbach and Douglas, 1989), and red clover (Martensson, 1992). However, with the exception of the study reported by Martensson (1992), the concentrations of chlorsulfuron at which reductions in nodulation were reported, were in excess of those used in the study in this thesis. Imazethapyr (200 ml ha^{-1}) also reduced the nodule numbers of *M. truncatula* cultivars Caliph, Mogul and Paraggio (Fajri *et al.*, 1996). However, again, the concentration of imazethapyr used by Fajri *et al.* (1996) for medic, was higher than that used for chickpea in this study.

The number of nodules on chickpea plants was also reduced by pre-exposing rhizobia cultures to chlorsulfuron or imazethapyr prior to inoculation, when no other herbicide was present (i.e. at germination or in the soil). The rhizobia were exposed to the recommended application rates of chlorsulfuron or imazethapyr in liquid culture, as natural rhizobial populations in the soil would undergo short term exposure to these concentrations upon application of the herbicides. In the case of imazethapyr, the reduction in nodulation due to rhizobia pre-exposure appears to be an infection problem, as nodule number, but not weight, was affected. When rhizobia were pre-exposed to chlorsulfuron, there was a reduction in both nodule number and weight, indicating a variation in the influence of imazethapyr and chlorsulfuron on the nitrogen fixation process. The nodules formed on chickpea plants, inoculated with rhizobia pre-exposed to chlorsulfuron, were capable of fixing more nitrogen per unit of nodule weight than those inoculated with non pre-exposed rhizobia. It was possible that the reduction in nodule number and weight could be offset by an increase in the amount of nitrogen fixed per unit of nodule weight. This was supported by the study of Herdina and Silsbury (1990), who reported that when half the nodules of a faba bean plant were removed, there was an increase in the specific activity of the remaining nodules. However, in this study, pre-exposing the rhizobia to imazethapyr did not affect the amount of plant nitrogen produced per unit of nodule weight. Therefore, the reduction in plant nitrogen was due to a reduction in the number of nodules rather than the nodules' ability to fix nitrogen. The differences observed between chlorsulfuron and imazethapyr in the amount of nitrogen fixed per unit of nodule weight, may be due to the fact that, although the number of nodules was reduced by pre-exposure of rhizobia to imazethapyr, nodule weight was not reduced. The reduction in nodulation due to pre-exposing rhizobia to imazethapyr or chlorsulfuron has not been reported prior to this

study. It is possible that the reductions are unique to the symbiosis between chickpea and their rhizobia. Pre-exposure of rhizobia to chlorsulfuron or imazethapyr may reduce numbers of nodules by several mechanisms including:

i) the herbicides directly reducing the growth or survival of rhizobia;

ii) herbicide carryover on rhizobial cells to the site of action inside root hairs even after rinsing or;

iii) by influencing the nodule infection and formation process.

Chlorsulfuron, imazethapyr or flumetsulam did not affect the growth, or doubling time, of rhizobia in *in-vitro* growth inhibition studies (Chapter 9). Generally rhizobia are unaffected by ALS-inhibiting herbicides, except at high levels (Martensson, 1992; Eberbach and Douglas, 1989; Martensson and Nilsson, 1989) and it is unlikely that the reduction in nodulation observed from rhizobia pre-exposure in this study, can be explained by the influence of the herbicide directly on the growth of rhizobia.

Subsequent investigations (Chapter 9) showed that the amount of herbicide on rhizobial cells (after rinsing with Ringer's solution) carried to the point of infection was minimal and would be inadequate to inhibit either chickpea root growth or nodulation (in terms of reduced root biomass and fewer infection sites). These results imply that pre-exposure of rhizobia to chlorsulfuron or imazethapyr, in the absence of any other herbicide in the system, can impair the complex process of nodule infection and formation. It has not been possible, in this study, to establish the detailed mechanism involved (see section 10.3).

10.1.1 Key findings

- Flumetsulam had little effect on shoot biomass or yield of medic, or chickpea, in the field.

- Residues of chlorsulfuron and triasulfuron reduced shoot biomass of medic, but seed yield was unaffected.

- Residues of chlorsulfuron and in-crop applications of imazethapyr reduced shoot biomass and nitrogen fixation of chickpeas in the field.

- High residual levels of chlorsulfuron combined with recommended application rates of imazethapyr reduced grain yield of chickpea in the field.

- The number of nodules on chickpea roots were reduced by the presence of imazethapyr and residual levels of chlorsulfuron in the growth media in a pot trial. The reduction in nodulation was due to reduced root biomass and therefore fewer available infection sites.

- Pre-exposing rhizobia to imazethapyr or chlorsulfuron, prior to inoculation, reduced the number of nodules formed on chickpea roots. However, the herbicides differ in their effects on the amount of nitrogen fixed per unit of nodule weight. Pre-exposure of the rhizobia to the herbicides may directly impact on nitrogen relations of the plant through reduced nodulation, caused by initiation problems rather than lack of infection sites due to reduced root biomass.

- The reduction in nodulation from pre-exposing rhizobia to chlorsulfuron or imazethapyr was not due to the herbicides affecting rhizobial growth.

- The presence of herbicide on rhizobial cells, following the rinsing process was insufficient to cause the observed reduction in nodulation.

- The results suggest that the herbicide may be impacting on the nodule infection and formation processes (see section 10.3 for description of possible mechanism and suggested further research).

10.2 IMPLICATIONS OF RESULTS

The reduction in *M. rugosa* shoot biomass from residues of chlorsulfuron and triasulfuron has the potential to reduce feed availability for grazing stock, which in turn, has implications for grazing management practices when medic pastures are included in pasture/cereal rotations. As medic pastures in southern Australia fix on average 20 – 25 kg N tonne^{-1} of shoot biomass (Peoples and Baldock, 2001), any reduction in shoot biomass from herbicide application, or residues of herbicides, will lead to a reduction in the amount of nitrogen incorporated in the medic/cereal rotation.

The reduction in chickpea shoot biomass may result in a reduction in nitrogen returned to the soil. Two studies in NSW have found that chickpeas can potentially return 33 and 65 kg N ha^{-1} to the soil from above ground biomass (Armstrong *et al.*, 1997; Evans *et al.*, 1989). As the turnover of roots and nodules is believed to provide mineralisable nitrogen for subsequent crops (Unkovich *et al.*, 1997; Peoples *et al.*, 1995b), the reduction in root biomass and nodulation also leads to a possible loss of nitrogen resources. Reduced nodulation due to the presence of chlorsulfuron or imazethapyr in the soil, or during the growth of rhizobia, has potential consequences for nitrogen fixation and the subsequent nitrogen balance of the soil. If the plant cannot

satisfy its own nitrogen requirements, it will utilise soil mineral nitrogen reserves (if available) and consequently reduce the nitrogen balance of the soil.

Crop legumes can fix between 6 to 97% of their nitrogen which equates to 29 - 348 kg N ha^{-1} (Peoples *et al.*, 1995a, b and c). Through fixation and the return of nitrogen rich plant residues to the soil, legumes have the potential to increase soil total nitrogen (Unkovich *et al.*, 1994; Peoples *et al.*, 1992; Evans *et al.*, 1989). Lupins in NSW and WA contributed an average of 40.3 kg N ha^{-1} (range – 41 to 135 kg N ha^{-1}) (Evans *et al.*, 1989) and 65 kg N ha^{-1} (range 32-96 kg N ha^{-1}) (Unkovich *et al.*, 1994) respectively to soil nitrogen. The studies in this thesis, found that chlorsulfuron reduced the amount of nitrogen fixed by chickpea plants by up to 55% in the field and 70% in a pot trial, relative to plants not exposed to the herbicide. Imazethapyr reduced the amount of nitrogen fixed by chickpea plants by up to 71% in the field and 96% in a pot trial. In the field, the combination of high concentrations of chlorsulfuron residues and in–crop application of imazethapyr reduced nitrogen fixed by 87%. ALS-inhibiting herbicides can therefore reduce the amount of nitrogen fixed by chickpeas and adversely affect the nitrogen balance of the soil. The reduction in nitrogen fixation increases the potential cost to the farmer, as any nitrogen loss would need to be replaced by inorganic nitrogen fertiliser applications to subsequent crops. Additional costs associated with the use of ALS-inhibiting herbicides may also be experienced from chickpea yield reductions demonstrated with high residual rates of chlorsulfuron and in-crop application of imazethapyr.

The results of the field trial in Chapter 5 (Table 10.1) and mean values from the Australian literature (Table 10.2) were used to calculate potential costs to the farmer, in terms of yield and nitrogen fixation reductions, from chlorsulfuron and imazethapyr use.

The nitrogen balance (N balance) of the soil following chlorsulfuron (3 g ai ha^{-1} or 20% of the recommended application rate), imazethapyr (29 g ai ha^{-1}) and the combination of chlorsulfuron and imazethapyr, was 80, 116 and 96% lower than the controls with no herbicide (Table 10.1). The reduction in N balance, following imazethapyr application, would require nitrogen fertiliser inputs of up to 29 kg N ha^{-1} to bring the N balance back to that where no herbicides were present (Table 10.1). The cost of adding this additional nitrogen in the form of urea equates to \$27.16 ha^{-1} but is far outweighed by the increase in yield and associated financial benefits (Table 10.1). The combination of high (20%) chlorsulfuron residues and imazethapyr resulted in reduced yield and a negative N balance of the soil, leading to potential costs to the farmer of up to \$54 ha^{-1}, in nitrogen replacement and yield loss, and a potential total cost to the Australian chickpea industry of \$11,850,090 (Table 10.1). The potential cost to the Australian chickpea industry represents the worst case scenario and assumes that: (i) the entire chickpea area is treated with chlorsulfuron prior to sowing chickpeas; (ii) high residues of chlorsulfuron remain over the entire area of chickpeas; and (iii) imazethapyr is sprayed on the entire chickpea crop. In highly alkaline soils, where sulfonylurea degradation by hydrolysis is slow, minimum plant back periods should be strictly followed, before sowing chickpeas, especially if weeds are to be controlled by imazethapyr.

The higher yield of crops treated with imazethapyr or high residual levels of chlorsulfuron may have been due to better weed control in comparison to the control plots. Additionally, rainfall late in the season allowed herbicide affected crops to recover and mature much later than the control plots. As the yields from the control plots in this study were low in comparison to literature values, another theoretical

estimate of the cost to farmers of herbicide use was calculated using average yields and nitrogen fixation data from the Australian literature (Table 10.2). In this scenario, the biologically fixed nitrogen control was the literature mean for chickpeas, and the percentage reduction in nitrogen fixed from chlorsulfuron (3 g ai ha^{-1}), imazethapyr or chlorsulfuron (3 g ai ha^{-1}) + imazethapyr, observed from the results in Chapter 5, were used to determine herbicide damage. The control yield value was also taken from means for Australian literature (Table 10.2). As yield was probably influenced by late rainfall in the field trial in Chapter 5, potential yield reductions from herbicide damage of 25, 50 and 70% were calculated for each herbicide and related reductions in nitrogen fixed (Table 10.2). In this way, it was possible to calculate theoretical farmer costs from the use of these herbicides. If chickpeas were sown in a field with high residual levels of chlorsulfuron, with imazethapyr applied to control weeds in that season, and a 25% reduction in yield was observed, up to 35 kg N ha^{-1} of fertiliser would be required to return the soil N balance to that of the control (Table 10.2). In this scenario, the overall cost in terms of yield loss and nitrogen fertiliser would be up to $167 per hectare for the farmer and $36.5 million to the chickpea industry (Table 10.2). Due to reduced nitrogen removed in grain, the N balance of herbicide treatments was often higher than those without herbicide. However, as the potential yield losses increase, so too does the potential cost to the farmer and the chickpea industry (Table 10.2).

Due to the potential cost in terms of soil N balance and yield, farmers may need to look at using alternatives to ALS-inhibiting herbicides, particularly on alkaline soils. Fromm (1996), suggested some alternative herbicides for the sulfonylureas, chlorsulfuron and triasulfuron. These alternative herbicides include trifluralin, diuron, bromoxynil/MCPA mixtures and dicamba (Fromm, 1996). Some of these products

however, may be more expensive than the relatively low cost sulfonylureas, particularly if both grasses and broadleaf weeds need to be controlled (Fromm, 1996). Where residues of sulfonylureas persist, in the alkaline soils of southern Australia, the additional cost of these alternative herbicides may outweigh the cost to the farmer from the potential damage to pastures or sensitive crops in terms of biomass, yield and N balance. However, the effect of these alternative herbicides on the symbiosis between rhizobia and legumes is also largely unknown. Other means of controlling weeds may also be examined including reducing the soil weed seed bank prior to sowing the crop, burning, grazing and cultivation (Fromm, 1996; Powles *et al.*, 1996).

Table 10.1: Potential cost of chlorsulfuron residues (3 g ai ha^{-1} or 20% of the recommended application rate), imazethapyr (29 g ai ha^{-1} or recommended application rate) or chlorsulfuron (3 g ai ha^{-1}) + imazethapyr (29 g ai ha^{-1}) in terms of yield and soil N balance of chickpeas based on data collected in a field trial presented in Chapter 5. + /- indicate a profit/loss to the farmer or chickpea industry.

	Herbicide			
	Nil	Chlorsulfuron	Imazethapyr	Chlorsulfuron + imazethapyr
Biologically fixed nitrogen (kg N ha^{-1})	31	14	9	4
Grain yield (kg ha^{-1})	165	264	370	86
Grain N (%)*	3.5	3.5	3.5	3.5
N removed in grain (kg N ha^{-1})	5.8	9.1	13	3
N balance kg N ha^{-1} (N returned to soil after chickpea)	25.2	4.9	-4	1
N (kg N ha^{-1}) required to eliminate cost of herbicide on N balance	0	20.3	29.2	24.2
Cost ($) of nitrogen fertiliser**(@ \$0.93 kg^{-1}) required to make up the N balance		18.88	27.16	22.51
Cost (\$ ha^{-1}) from loss in yield*** (@ \$0.40 kg^{-1})		+39.60	+82.00	-31.60
Total profit/loss (\$ ha^{-1}) to the farmer		+20.72	+54.84	-54.11
Total profit/loss ($) to Australian chickpea industry (219,000 ha in 1997)		+4,537,680	+12,009,960	-11,850,090

*A grain N of 3.5% was found for seeds not treated with herbicide and was chosen as the constant value in these calculations as grain N was not calculated for herbicide treated plants.

**The cost of nitrogen fertiliser (urea) was obtained from Pivot Ltd and was based on urea consisting of 46% N at \$0.43 kg^{-1}.

*** The price of chickpeas (\$400 $tonne^{-1}$) was obtained from the Stock Journal (2001).

Table 10.2: Potential cost of chlorsulfuron (3 g ai ha^{-1}), imazethapyr (29 g ai ha^{-1}) or chlorsulfuron + imazethapyr applied to chickpea crops in terms of yield and soil N balance. The nil biologically fixed N and yield values are mean Australian literature values for chickpeas with the range of data shown in parentheses. The biologically fixed N for herbicide treatments are based on the % reduction observed from these treatments in the field trial in Chapter 5. The theoretical cost was calculated in terms of potential yield losses of 25, 50 and 70%.

	Herbicide								
	Nil	Chlorsulfuron			Imazethapyr			Chlorsulfuron + imazethapyr	
Biologically fixed N (kg N ha^{-1})	54.2 (12.6 –104)*	33.6			16.26			7.05	
Grain yield (kg ha^{-1})		25%	50%	70%	25%	50%	70%	25%	50%	70%
	1340** (570 – 3240)	1005	670	402	1005	670	402	1005	670	402
N removed in grain (kg N ha^{-1}) with 3.5% grain N	46.9	35.18	23.45	14.07	35.18	23.45	14.07	35.18	23.45	14.07
N balance (N returned to soil following chickpea crop)	7.3	-1.58	10.15	19.53	-18.92	-7.19	2.19	-28.13	-16.40	-7.02
N required to eliminate cost of herbicide on N balance		8.88	+2.85	+12.23	26.22	14.49	5.11	35.43	23.70	14.32
Cost ($ ha^{-1}) of nitrogen fertiliser (@ $0.93 kg^{-1}) required to make up the N balance		8.26	(+2.65)	(+11.37)	24.38	13.47	4.75	32.95	22.04	13.32
Cost ($) from loss in chickpea yield (@$0.40 kg^{-1})		134	268	375.2	134	268	375.2	134	268	375.2
Total cost ($ ha^{-1}) to the farmer		142.26	265.35	363.83	158.38	281.47	379.95	166.95	290.04	388.52
Total cost ($) to Australian chickpea industry (219,000 ha Siddique and Sykes, 1997)		31,154,940	58,111,650	79,678,770	34,685,220	61,641,930	83,209,050	36,562,050	63,518,760	85,085,880

*Taken from: Armstrong *et al.*, 1997; Horn *et al.*, 1996b; Herridge *et al.*, 1995; Doughton *et al.*, 1993; Evans *et al.*, 1989. **Taken from Australian literature: Armstrong *et al.*, 1997; Horn *et al.*, 1996a; Herridge *et al.*, 1995; Thomas and Fukai, 1995; Doughton *et al.*, 1993; Siddique *et al.*, 1993; Siddique and Pritchard, 1993; Beech and Leach, 1989.

10.3 FUTURE WORK

10.3.1 Nodule formation and infection

Observed results from the studies reported in this thesis suggested that pre-exposure of rhizobia to chlorsulfuron or imazethapyr inhibited the ability of rhizobia to infect plant root hairs or form nodules. This reduction in the number of nodules was not due to direct herbicide effects on the rhizobia themselves or herbicide carryover on rhizobial cells. In order to achieve successful nodulation, *Rhizobium* must be present in free living form in the rhizosphere (Richardson *et al.*, 1989). Natural populations of rhizobia in the soil may come into contact with ALS-inhibiting herbicides, when the herbicides are applied to the soil. If chlorsulfuron or imazethapyr are having an effect on the ability of chickpea rhizobia to infect plant root hairs, it will have consequences for subsequent chickpea crops, particularly if the farmer does not inoculate the chickpea seeds at the time of sowing. This may inhibit the ability of the symbiosis between the plant and rhizobia, to form nodules and thus reduce nitrogen fixation. The process of nodule formation was discussed in Chapter 2, section 2.6.2 and Figure 2.1, but additional details are discussed here.

The 'common' nod genes, *nodABC,* are essential for nodulation to occur (Suominen *et al.*, 1999; Hungria and Stacey, 1997; Dénarié *et al.*, 1996; Fisher and Long, 1992) and are clustered on the Sym plasmid (Richardson *et al.*, 1989). When Sym plasmids are removed from many strains of rhizobia, the resulting mutant cells are unable to interact with their respective legume hosts and nodules do not form (Rolfe and Richardson, 1987). The *nodD* gene is a regulatory gene that regulates gene transcription (Hungria and Stacey, 1997). The *nod* genes are involved in the synthesis

of Nod factors that induce nodule formation in legumes (vanRhijn and Vanderleyden, 1995). The Nod factors were found to induce cortical cell divisions that later formed the nodule on lucerne seedlings (Fisher and Long, 1992). Mutations in the *nod* genes can lead to disturbances in the nodule formation process such as the failure of infection threads to form, or the formation of non fixing nodules (Hungria and Stacey, 1997; vanRhijn and Vanderleyden, 1995). It is possible that pre-exposure of rhizobia to chlorsulfuron interferes with the *nod* genes in some way. This may include a non-synthesis of Nod factors leading to failure of the cortical cells to divide and consequently failure of the nodule meristem to form. Pre-exposing rhizobia to chlorsulfuron may inhibit the ability of rhizobia to recognise specific flavonoid compounds, interfere with the Sym plasmid or stop root hair curling. If a rhizobia bacterium that was carrying chlorsulfuron entered the nodule meristem, it would carry the herbicide to its site of action and stop further cell division and growth. However, the quantity carried in this manner is not likely to have herbicidal effects on the root. It is possible that there may also be a combination of effects such as direct effects on the rhizobial nodulation factors, as well as direct effects on root biochemistry or signalling, that may occur even when no other herbicide induced damage to the plants is evident. Any of the above suggestions represents a possibility, and further work is required to determine why the pre-exposure of rhizobia to chlorsulfuron inhibits nodulation.

10.3.2 Suggested further work

Further experiments need to be conducted to investigate the impacts of these herbicides (chlorsulfuron and imazethapyr) on root hair infection and the nodule formation process. This could include investigating the:

- Effects of ALS-inhibiting herbicides on *nod* genes and gene expression. For example, *nod* genes are involved in the induction of nodules and it is possible that the herbicide can interfere with their function or expression resulting in failure of nodules to develop.

- Biochemistry of root signals. For example, interference with the production or recognition of flavonoids. When rhizobia attach to the root, the plant releases lectins and the rhizobia release polysaccharides and the herbicide may be impairing one of these processes.

- Effects on root curling. For example, the herbicide may actually interfere with the morphology of the root. Microscopic studies may help to answer this question.

- An Ames test may help to determine if the ALS-inhibiting herbicides investigated in this study are causing genetic damage, leading to gene mutations (Charles *et al.*, 2000; Mortelmans and Zeiger, 2000).

- Assess if the impacts of ALS-inhibiting herbicides on chickpea/rhizobia nodulation and symbiosis are also observed in other grain legume or pasture symbioses.

10.4 SUMMARY

The results of the study in this thesis have suggested that caution needs to be taken when using ALS-inhibiting herbicides, not only due to reduction in plant biomass, yield and nitrogen fixation, but because of the hidden cost of pre-exposing rhizobia to

chlorsulfuron and imazethapyr. This pre-exposure of rhizobia to chlorsulfuron or imazethapyr led to fewer nodules, which may in turn have led to a reduction in nitrogen fixation. Herbicide manufacturers, in the future, may need to undertake more stringent testing, not only on herbicide effects on plants and rhizobia, but also on their symbiosis. Overall, the use of chlorsulfuron and imazethapyr may lead to an inefficient use of a legume in a cropping sequence, and an inadvertent increase in financial cost to the farmer. More work needs to be undertaken to determine the mechanism by which pre-exposure of rhizobia to chlorsulfuron or imazethapyr is inhibiting nodulation.

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