The impact of acetohydroxyacid synthase inhibiting herbicides on symbiotic nitrogen fixation of grain and pasture legumes

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Acronyms and abbreviations

a.i.	active ingredient
A500	absorbance at 500nm
AA	amino acid
AHAS	acetohydroxyacid synthase
AHASIH	acetohydroxyacid synthase inhibiting herbicide
ALS	acetolactate synthase
ANOVA	analysis of variance
AOX	alternative oxidase
BCAA	branched chain amino acid
BNF	biological nitrogen fixation
CFU	colony forming units
CS	chlorsulfuron
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
F	flumetsulam
FAD	flavin adenine dinucleotide
GST	glutathione s-transferase
IM	imazethapyr
IX	imazamox
LSD	least significant difference
MM	metsulfuron methyl
N	nitrogen
N_2	molecular nitrogen
[N]	nitrogen concentration
na	not applicable
ns	not significant
PAL	phenylalanin ammonia-lyase
PBS	phosphate buffered saline
ppm	parts per million
PSPE	post-sowing pre-emergence
RBP	RuBisCO binding protein
REML	restricted maximal likelihood
RNA	ribonucleic acid
rpm	revolutions per minute
RuBisCO	ribulose bisphosphate carboxylase
SU	sulfonylurea
TAL	tyrosine ammonia-lyase

Scientific and common names

Legumes	
Cicer arietinum	chickpea
Glycine max	soybean
Lens culinaris	lentil
Lupinus albus	lupin
Medicago littoralis	strand medic
Medicago sativa	lucerne, alfalfa
Medicago truncatula	barrel medic
Ornithopus compressus	serradella
Pisum sativum	field pea
Trifolium michelianum	balansa clover
Trifolium subterraneum	subterraneum clover
Vicia faba	faba bean
Vicia sativum	vetch
Other plants	
Amsinckia intermedia	burrweed
Arachis hypogaea	peanuts
Capsella bursa-pastoris	shepherd's purse
Carthamus lanatus	thistle
Erodium spp	storksbill
Galium tricornutum	three-horned bedstraw
Juncus bufonius	toad rush
Lathyrus	lathyrus
Lemna minor	duckweed
Rapistrum rugosum	wild turnip, turnip weed
Raphanus raphanistrum	wild radish
Sisymbrium orientale	Indian hedge mustard
Trigonella foenum-graecum	fenugreek
Urtica incisa	nettle
Zea mays	maize

Abstract

Group B herbicides inhibit the acetohydroxyacid synthase (AHAS - also known as acetolactate synthase) enzyme in the pathway of branched chain amino acid synthesis. These herbicides have gained widespread use in Australia, however potential impacts on nitrogen fixation by legumes have not been comprehensively assessed. Group B herbicides recommended for in-crop application to grain and pasture legume species were assessed for impacts on growth, nodulation and nitrogen fixation. Although it was demonstrated that nitrogen fixation can be affected by these herbicides, the range of responses indicated that multiple mechanisms could be responsible. These could include a reduction nitrogen fixation directly coupled to reduced plant growth; more specific and direct disruption of nitrogen fixation related to the inhibition of nodulation; or other mechanisms yet to be defined that could include affects on the rhizobia. To begin to understand these mechanisms, a herbicide tolerant *Medicago littoralis* cultivar 'FEH-1' was compared to Herald. Decreased nodulation, nitrogen fixation and acetylene reduction activity due to herbicide application were primarily related to the susceptibility of the plant to the herbicide. Thus herbicide tolerant legumes have the potential to alleviate suboptimal nitrogen fixation due to group B herbicides. A proteomics study of the response of root tips of model legume *Medicago truncatula* A17 to flumetsulam and metsulfuron methyl was conducted to identify more specifically the herbicide impacts on plant physiology. An increased abundance of stress response proteins and a decline in the abundance of some metabolic proteins was found, including a reduction in the abundance of glutamine synthetase which is expected to have direct consequences for the regulation of nitrogen fixation. Observations of root morphology revealed changes to root hairs and the development of lateral roots related to the disruption of meristems, with likely consequences for infection and nodule development. The results from this thesis confirm the potential for acetohydroxyacid synthase inhibiting herbicides to reduce nitrogen fixation of legumes. In addition to a general effect on nitrogen fixation via coupling to reduced plant growth, more specific biochemical and morphological mechanisms that disrupt nodulation are plausible.

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

Ryan Farquharson

July 2009

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Adelaide Creek – I liked it so much I decided to stay.

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Told you I'd finish.

The Prescott group (an outlet for healthy cynicism)
Various flat mates – the good (Therese, Karen, Jon), the brotherly (Jean-Patrick), the welcoming (Myrna) and the insane.

The Fernandez family for a home away from home.

And last but not least, my family.

To my wife.

They told us to collaborate…

And we did.

Chapter 1 Introduction

Nitrogen is an essential component of amino acids and proteins, and is therefore one of the most important elements for the establishment and maintenance of life. Despite the abundance of molecular nitrogen (N_2) in the atmosphere and the earth's crust, nitrogen is a nutrient that is often limiting to plant growth. This is because N_2 is a relatively inert molecule and most organisms require combined or 'fixed' forms of nitrogen. The enzyme complex that catalyses the reduction of N_2 to ammonia is called nitrogenase, and only certain microorganisms called diazotrophs possess it. Diazotrophs are loosely divided into free-living and symbiotic forms, with symbiotic nitrogen fixation generally considered the most productive terrestrial form of biological nitrogen fixation (BNF). The legume / *Rhizobium* symbiosis is particularly important in sustaining crop and pasture production.

Increasing yield potential and maximising productivity has been the driving force behind farming system management in the past. This aim is now combined with the desire for sustainability (Peoples *et al*., 1995). Farms must become balanced systems, replenishing reserves of nutrients that are removed or lost from soils. Inputs of nitrogen are in the form of nitrogenous fertilisers or biologically fixed nitrogen. In recent decades, increasing amounts of fertiliser N have been used, diminishing the role for BNF (Peoples *et al*., 1995). However BNF by legumes is still very important in Australian agriculture, and will continue to be a focus due to its potential to sustain crop and pasture production (Turner, 2001). Fossil fuels are a finite resource, the combustion of which is responsible for the majority of anthropogenic global warming. As the cost of fossil fuels and hence nitrogenous fertilisers becomes volatile and ultimately more expensive, biologically fixed nitrogen is likely to have a larger role in agriculture in the future. Other anthropogenic sources of greenhouse gases include nitrous oxide from the nitrification and denitrification of fertiliser nitrogen. The supply of nitrogen to agricultural systems from legumes has the potential to reduce excess amounts of inorganic N and hence nitrous oxide emissions from agriculture.

Australian farmers have exploited annual pasture legumes since the early 20th century to provide high quality food for grazing animals and to improve soil N fertility for cereal production in long-term rotations (Unkovich *et al*., 1997). Legumes contribute to

the soil N balance by providing low C:N residues which may be mineralised and release inorganic N in the soil. In recent decades, a range of crop legumes have been introduced to the extent that both grain and pasture legumes now play a key role in rotations with cereals and broad-leafed crops in many regions of southern Australia (Unkovich *et al*., 1997). The principle legumes used in southern Australia range from extensive pastures where clovers, medics, lucerne, serradella and biserrula are important; to intensive grain production using legume crops such as beans, peas, lentils, chickpeas and lupins.

Apart from the benefits to soil N status, there are a number of other advantages of using legumes in agricultural systems (Peoples and Crasswell, 1992). When used in rotation with cereals, legumes may provide a break to cereal disease and pest cycles. Legumes also provide increased options for weed control. If weeds are not controlled, crop yields are reduced, harvest operations are impeded, produce is contaminated and infestation of following cereals can occur. A number of herbicides are licensed for use in legumes.

Farmers and agronomists are concerned about the effects of the group B or acetohydroxyacid synthase inhibiting herbicides (AHASIH) on legumes generally, but with a particular emphasis on nitrogen fixation. Stunting of growth and yellowing of leaves has been observed following application of recommended AHASIH to legumes, or when legumes have been grown subsequent to cereals in which the sulfonylurea class of group B herbicides have been used. Researchers and agronomists have also observed changes in nodule appearance post herbicide application. Variety trials that test the suitability of herbicides on legumes usually only measure effects on yield. As a result, warnings about symptoms and minimum plant back times appear on herbicide labels along with the statement that yields are unaffected. In addition to yield penalties, it is vital to know what effect AHASIH may be having on the nitrogen fixation by legume crops and pasture species to gain a full understanding of the implications on soil N status and subsequent requirements to optimise N delivery to growing crops.

The group B or acetohydroxyacid synthase (also known as acetolactate synthase) inhibiting herbicides inhibit the first enzymatic step in branched chain amino acid biosynthesis. As of the year 2000 there were five classes based on chemical structure and 55 different active ingredients commercialised worldwide, with more in

development (see Chapter 2). The most relevant chemicals in the three major classes (sulfonylureas, imidazolinones and triazolopyrimidine sulfonamides) are described later in Chapter 2. Since their introduction in the early 1980's, group B herbicides have gained widespread usage in southern Australian farming systems (Heap, 2000). They are used in almost every crop. The group B herbicides are favoured because of their potency, low mammalian toxicity, broad weed control spectrum, and relatively low cost (Beyer *et al*., 1988).

The general visual symptoms of nitrogen deficiency were described by Wallace (1961). They include "restricted growth of both tops and shoots; shoots are short and thin, and growth is upright and spindly; leaves are small, usually of pale, yellowish green colour in the early stages of growth, and later they develop highly coloured tints of yellow, orange and red, or sometimes purple; tinting begins on the older foliage and proceeds towards the younger leaves, and the tints may also be developed in the petioles; defoliation is premature and begins at the older leaves; lateral shoots are few and lateral buds may die or remain dormant; blossoming in severe cases is greatly reduced and consequently yields of grain and fruits are very small." Similar symptoms have been observed on legumes exposed to group B herbicides and raise the question of whether they are indicative of a reduced ability to fix nitrogen.

There are four aspects that provide a useful framework with which to consider the possible impacts of AHASIH on symbiotic nitrogen fixation (Eberbach, 1993). Herbicides can impact upon:
(1) the legume symbiont; or
(2) the rhizobia symbiont; or
(3) the interactions between the symbionts; or
(4) the nitrogen fixation process itself.
These are discussed in Chapter 2. In addition, extraneous factors or stressors that ordinarily impact on nitrogen fixation may be exacerbated by AHASIH.

Legumes are unique in that they can obtain combined nitrogen from their symbiosis with rhizobia. Potential reductions in symbiotic nitrogen fixation may not be obvious and are difficult to assess in the field. Since nitrogen fixation by the legume / *Rhizobium* symbiosis is a key reason for growing legumes in southern Australian

agriculture, it is vital that we ascertain the impact AHASIH may be having in this regard. An understanding of the mechanisms of any impacts may assist farmers in decisions they make with respect to herbicide strategies and soil fertility.

1.1 Aims

The work described in this thesis aims to assess the potential for recommended group B herbicides to inhibit nitrogen fixation by important grain and pasture legumes grown in southern Australia. The second aim is to better understand the mechanisms involved.

1.2 Thesis structure

This thesis is structured as per Figure 1.1. A review of the literature current to the start of candidature (2002) is provided in Chapter 2. In Chapter 3 and 4, screening experiments of grain and pasture legumes (respectively) is presented. Chapter 5 discusses both sections of screening work. To start to determine mechanisms, an experiment comparing Herald with a herbicide tolerant form of *Medicago littoralis* is presented in Chapter 6. In Chapter 7 the response of the root morphology and proteome of *Medicago truncatula* two group B herbicides is examined *in vitro*. Chapter 8 synthesizes the results of the experimental work, discusses potential mechanisms in context with the literature and highlights areas for future research.

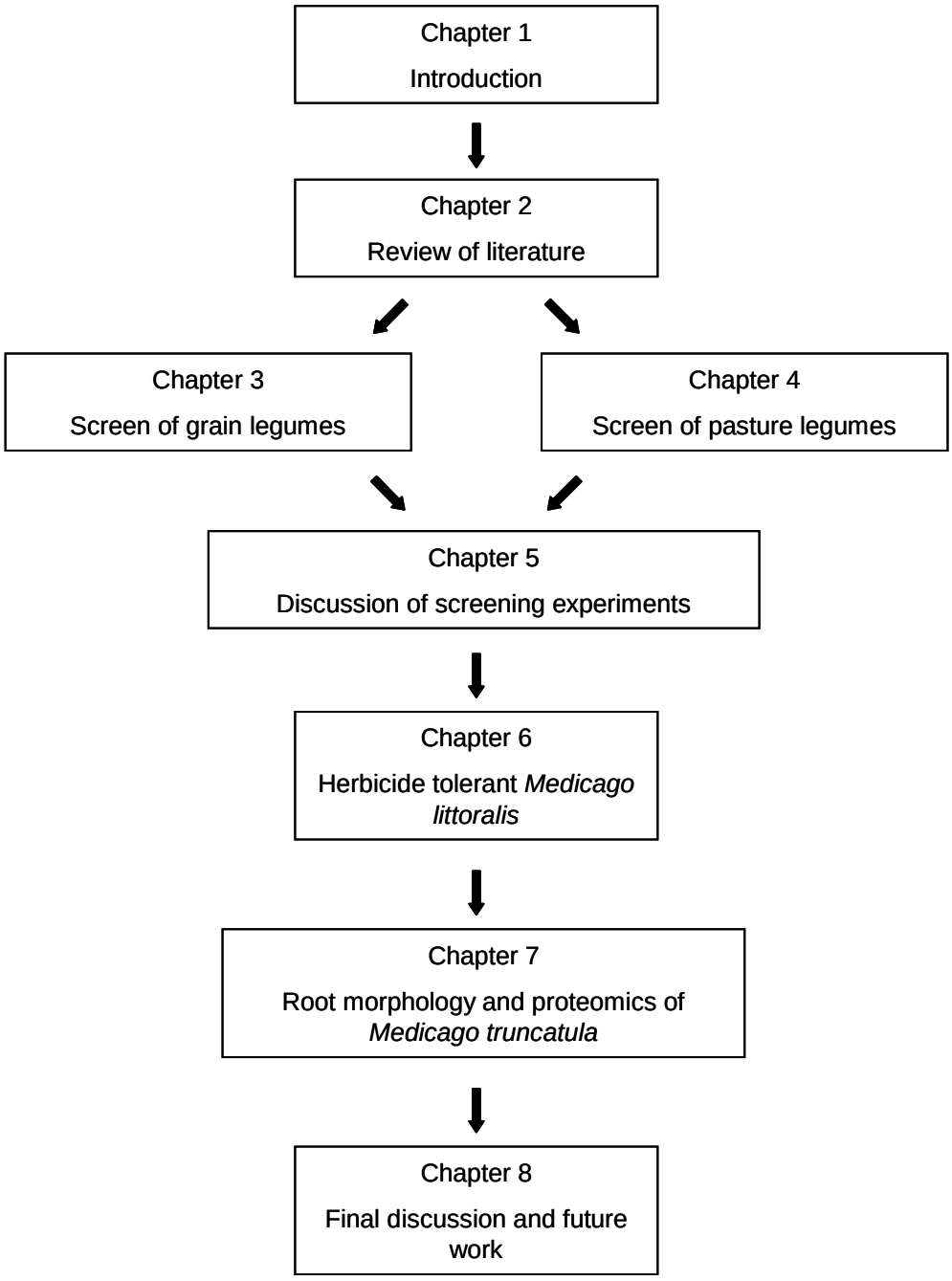

Figure 1.1 Thesis structure

Chapter 2 Literature review

2.1 Introduction

Based on the literature published prior to the commencement of this project in 2002, this chapter provides a background on the group B or acetohydroxyacid synthase inhibiting herbicides (AHASIH), with particular reference to their impact on symbiotic nitrogen fixation by pasture and grain legumes. Possible mechanisms of inhibition include herbicide impacts on the plant, the rhizobia, the symbiosis and on nitrogen fixation itself. These mechanisms are discussed in the context of the available literature.

Residual and in-crop group B herbicides can reduce the growth and amount of nitrogen fixed by legumes. The formation of the legume / *Rhizobium* symbiosis is a complex process. Hence there are many possible ways in which these herbicides can impact on

nitrogen fixation. Most research indicates that the host plant is more susceptible than the rhizobia since growth of rhizobia exposed to the herbicides is generally not affected. However bacteria including rhizobia possess the herbicide target enzyme (acetohydroxyacid synthase), and the possibility that the ability of rhizobia to form a symbiosis is disrupted independent of effects on cell growth should be explored. The nitrogen fixation process itself is likely to be affected indirectly. Reductions in nitrogenase activity have been demonstrated but the mechanisms remain to be elucidated. The introduction of crop and pasture species resistant to AHASIH is likely to encourage greater use of these herbicides. It is therefore vital that the impact of these herbicides on nitrogen fixation is thoroughly understood given the important role of legumes in nitrogen supply and cycling in Australian farming systems.

2.1.1 Weed control

Weed control is crucial for maximising crop yields through reduced competition for water and nutrients and preventing the contamination of produce. Weed control can be executed by using crop and pasture rotations, grazing, cultivation and the application of herbicides (Powles *et al*., 1996). Herbicides enable cropping with minimum tillage, which is important for reducing soil compaction and erosion and for the conservation of soil organic matter.

There are numerous classes of herbicide based on their mode of action (Figure 2.1). Since their introduction in the early 1980's, group B herbicides have gained widespread usage in southern Australian farming systems (Heap, 2000). They are used in almost every broad acre crop. The group B herbicides are favoured because of their potency, low mammalian toxicity, broad weed control spectrum, ease of handling and relatively low cost (Beyer *et al*., 1988).

Figure 2.1 Herbicides by class (Go to www.plantprotection.org/hrac for full size poster)

2.1.2 Description of group B herbicides

The group B or acetohydroxyacid synthase (also known as acetolactate synthase) inhibiting herbicides (AHASIH) inhibit the first enzymatic step in branched chain amino acid (BCCA) biosynthesis. In 2000 there were five classes based on chemical structure and 55 different active ingredients commercialised worldwide, with more in development (Table 2.1). The chemicals most relevant to legumes in the three major classes (sulfonylureas, imidazolinones and triazolopyrimidine sulfonamides) are described later in this section.

Table 2.1 Group B (acetohydroxyacid synthase inhibiting) herbicides (Weedscience)

NOTE:
This table is included on page 31-32 of the print copy of the thesis held in the University of Adelaide Library.

Sulfonylureas

The sulfonylureas are the most potent inhibitors of plant growth available (Ferris *et al*., 1992). With application rates as low as 2 g ha^{-1} (Beyer *et al*., 1988), they have 100 to 1000 times lower application rates than some other herbicides. One advantage of sulfonylurea herbicides is thus improved handling, application and container disposal

activities. Sulfonylureas are generally recommended for use in cereal crops for the control of broadleaf weeds and some grasses. Sulfonylureas are not recommended for application to legume crops or pastures thus there are usually no in-crop implications of sulfonylureas for legumes. However extension of sulfonylureas to use in legumes has occurred. Chlorsulfuron (Figure 2.2) has been used for control of broadleaf weeds in lucerne crops in South Australia (Koopman *et al*., 1995). This practice has been discouraged, and farmers are becoming more aware of the impact of chlorsulfuron and other sulfonylureas such as triasulfuron on legumes.

The major concern about sulfonylureas affecting legume growth and nitrogen fixation is when sulfonylureas persist in the soil after application to cereals, and then legumes are subsequently used in rotation. The growing knowledge of the persistence of sulfonylureas has led to recommendations of generous plant-back times for legumes (e.g. 9 to 36 months), especially in areas with high pH soils and low rainfall. Persistence of group B herbicides in soil is dealt with in section 2.1.4.

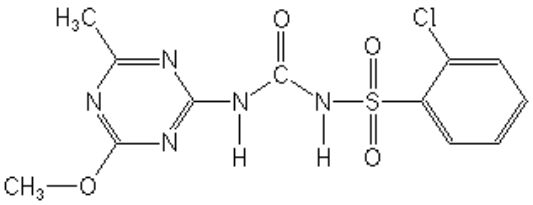

Figure 2.2 Chlorsulfuron chemical structure

Imidazolinones

The imidazolinones include imazethapyr, imazaquin, imazapyr and imazamethabenz-methyl (Shaner and O'Connor, 1991). More recent imidazolinones are imazamox and imazapic. Imazaquin has been used as a broad spectrum herbicide in soybeans (Bhalla *et al*., 1991). Imazethapyr and imazamox are recommended for use in a number of legume crops and pastures.

Imazethapyr (Figure 2.3) is used to control broadleaf weeds and grasses in soybeans and other leguminous crops (Hart *et al*., 1991). It is weakly acidic, with a pKa of the carboxylic acid group of 3.9 (Wepplo, 1991). Weeds controlled by imazethapyr as a pre-emergence application in Australia include burrweed (*Amsinckia intermedia*),

shepherd's purse (*Capsella bursa-pastoris*), thistle (*Carthamus lanatus*), storksbill (*Erodium* spp.), toad rush (*Juncus bufonius*), wild radish (*Raphanus raphanistrum*), Indian hedge mustard (*Sisymbrium orientale*), and nettle (*Urtica incisa*).

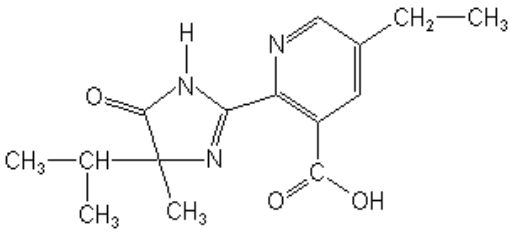

Figure 2.3 Imazethapyr chemical structure

Imazamox (Figure 2.4) is used to control weeds in field peas, subterranean clover and lucerne (Raptor® herbicide label). A more recent group B herbicide to be commercialised, imazamox is also being assessed for use in medic, balansa clover and serradella.

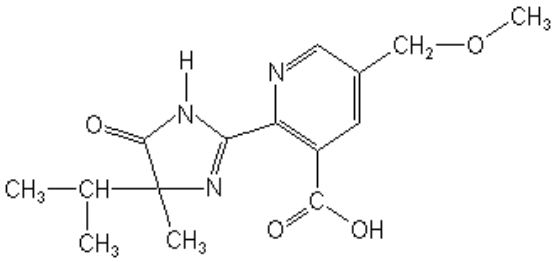

Figure 2.4 Imazamox chemical structure

Triazolopyrimidine sulfonamides

Flumetsulam (Figure 2.5) is the most widely used sulfonamide, manufactured by Dow Elenco and sold as Broadstrike. Broadstrike is registered for post-emergence control of certain broadleaf weeds such as wild turnip, Indian hedge mustard, three-horned bedstraw, amsinkia and turnip weed in (1) winter cereals (including those undersown with clover, lucerne or medics); (2) clover, fenugreek, lathyrus, lucerne, medicago and serradella seed crops and pastures; (3) chickpeas, field peas, lentils, maize, peanuts; and (4) for the pre-emergence control of certain broadleaf weeds in maize and soybeans. Flumetsulam is therefore the most important AHAS inhibiting herbicide recommended for use with legumes.

Flumetsulam is a weak acid with a pKa of 4.6. The anionic species is expected to prevail in alkaline soils, and the uncharged form could also exist in neutral to slightly acidic soils.

Metosulam is the only ALS inhibiting herbicide recommended for use in lupins.

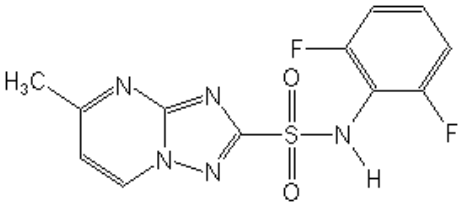

Figure 2.5 Flumetsulam chemical structure

2.1.3 Group B herbicide mode of action

Group B herbicides inhibit the second enzyme common to the synthesis of the branched chain amino acids valine, leucine and isoleucine. This enzyme is commonly known as acetolactate synthase (ALS), however there are actually two classes of enzymes that produce acetolactate. The term 'acetohydroxyacid synthase' (AHAS) is the more appropriate name for the enzyme inhibited by group B herbicides (Duggleby and Pang, 2000; Fryer and Makepeace, 1977).

2.1.3.1 ALS or AHAS?

The two classes of enzymes, acetolactate synthase or acetohydroxyacid synthase (AHAS) enzymes, each have their own physiological roles, substrate specificities, and cofactor requirements (Chipman *et al*., 1998). They exhibit about 30% homology (Maestri and Josef, 2000) but only AHAS is known to be inhibited by group B herbicides.

Catabolic ALS

Catabolic ALS is devoted to post-exponential growth phase production of acetoin in bacteria. These enzymes are not dependent on flavin adenine dinucleotide (FAD) (Stormer, 1968) and are not inhibited by the branched chain amino acids. They have a

low capability for acetohydroxybutyrate synthesis. They have relatively low pH optima and are hence sometimes called 'pH 6 acetolactate synthases,' and more recently α-acetolactate synthases.

The alsS protein from bacteria condenses two pyruvate molecules to form acetolactate, and is implicated in pH homeostasis via the acetoin - 2,3-butanediol pathway. A number of bacterial genera including *Enterobacter* (Stormer, 1967), *Bacillus* (Holtzclaw and Chapman, 1975), *Klebsiella* (Peng *et al*., 1992), and *Lactobacillus* (Tsau *et al*., 1992) are known to make use of the acetoin - 2,3-butanediol pathway, which contributes to the elimination of intracellular pyruvate (Maestri and Josef, 2000) as a mechanism for detoxification and pH homeostasis (Tsau *et al*., 1992). This mechanism for diverting glucose metabolism from acidic to neutral products occurs at low pH and oxygen limiting conditions when microbial cells enter the stationary phase of growth (Forlani *et al*., 1999). α-Acetolactate is the precursor of the aromatic C_4 compounds which are important for the aromatic qualities of fermented milk products (Cavin *et al*., 1999). In aerobic conditions, α-acetolactate is spontaneously decarboxylated to diacetyl, while under anaerobic conditions it is decarboxylated into acetoin (Cavin *et al*., 1999). The production of α-acetolactate from pyruvate will directly influence cell growth since it modulates the levels of pyruvate and coenzymes that can be used for anaerobic reactions (Cavin *et al*., 1999).

No evidence has been found for acetolactate derived acetoin production in plants as yet. Acetoin synthesis from pyruvate has been detected in maize, rice, tobacco and carrot suspension cultures, but this was attributed to a side reaction of pyruvate decarboxylase and a putative pyruvate carboligase (Forlani *et al*., 1999).

No evidence was found in the literature for the inhibition of catabolic ALS by group B herbicides. The AHAS enzymes that are known to be inhibited by herbicides belong to the anabolic class (described below).

Anabolic AHAS

Anabolic AHAS (reviewed by Duggleby and Pang (2000)) functions as the first common enzyme in the pathway for biosynthesis of the branched chain amino acids (BCAAs) valine, leucine and isoleucine (Figure 2.6). This enzyme is still commonly

referred to as acetolactate synthase, however two products are formed. AHAS catalyses the condensation of pyruvate and α-ketobutyrate to acetohydroxybutyrate in the pathway of isoleucine synthesis, or the condensation of two molecules of pyruvate to acetolactate in the pathway for leucine and valine synthesis. These enzymes are more appropriately referred to as acetohydroxyacid synthases (AHAS) because this name encompasses both of the physiologically important end products, acetohydroxybutyrate and acetolactate. The BCAA synthesis pathway is found in plants, bacteria, archae, fungi and algae, but not in animals.

AHAS enzymes are flavoproteins, requiring flavin adenine dinucleotide (FAD) and are thus sometimes referred to as FAD-dependent AHAS to distinguish them from the catabolic ALS which does not require FAD. Most are sensitive to feedback inhibition by one or more of the BCAAs and it is this class of enzymes that are inhibited by the group B herbicides. The rest of this section will focus on this class. The terms 'ALS' and 'AHAS' have been used interchangeably in the literature however in this review they are referred to as AHAS. A thorough review of AHAS, its structure, biochemical properties and herbicide binding has been published (Duggleby and Pang, 2000) thus only a basic discussion is provided here.

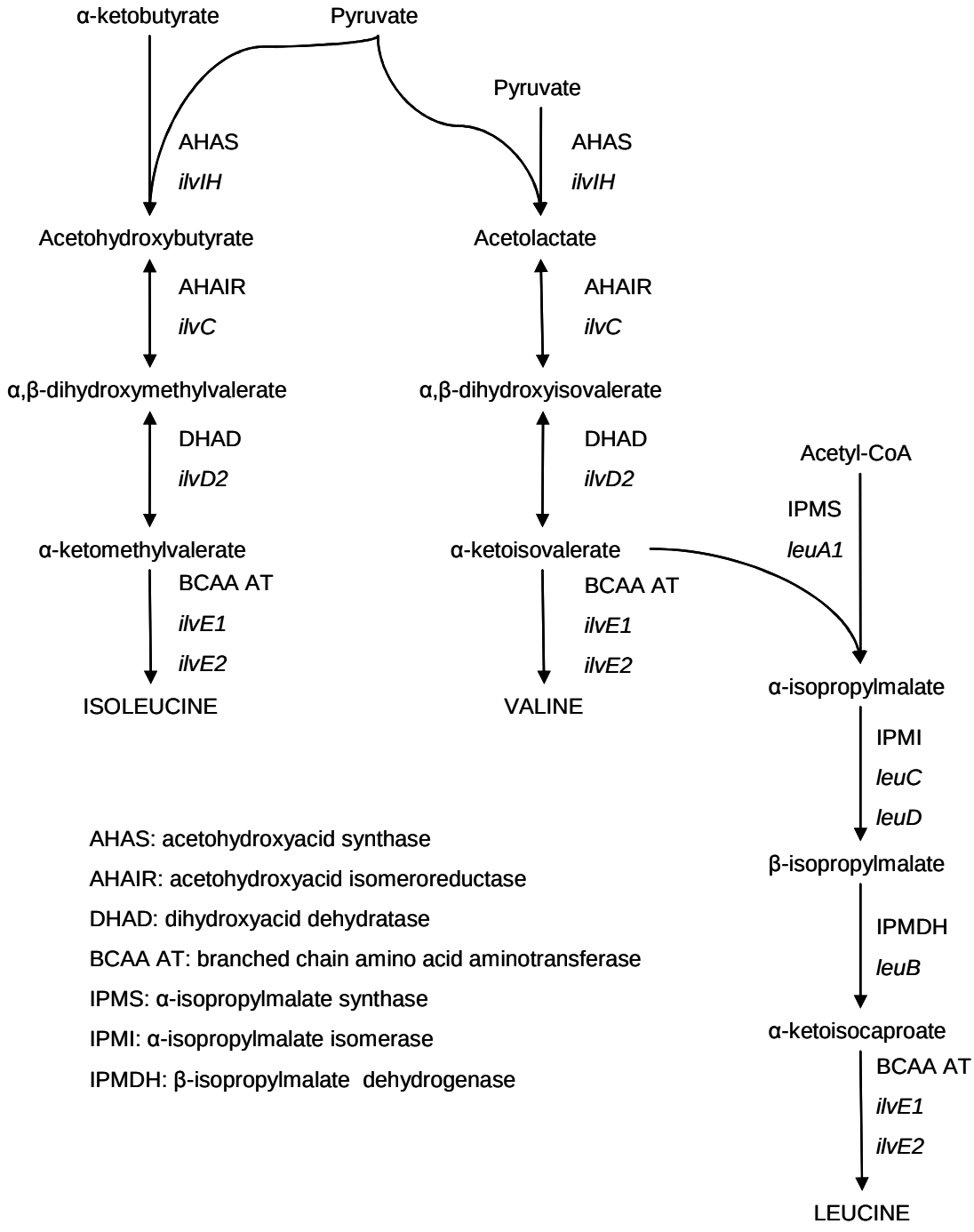

Figure 2.6: Branched-chain amino acid biosynthesis: enzymes, genes (italicised) and products

Bacterial AHAS

There are three different FAD-dependent AHAS enzymes in enterobacteria. Each isozyme consists of two catalytic subunits of about 60 kDa, and two smaller regulatory subunits (Table 2.2).

Table 2.2 ALS/AHAS isozymes in *E. coli* from Chipman *et al*. (1998)

Isozyme	Catalytic subunit size	Catalytic subunit gene	Regulatory subunit size	Regulatory subunit gene
I	60 400	IlvB	11100	IlvN
II	59300	IlvG	9700	IlvM
III	61800	IlvI	17500	IlvH

All three isozymes are expressed in natural isolates of *Escherichia coli* and *Staphylococcus typhimurium*. Expression of the three isozymes is regulated differently, and they have complementary physiological roles (Chipman *et al*., 1998). Under appropriate growth conditions, one isozyme is sufficient for non-auxotrophic growth. Indeed, most prokaryotes whose genomes have been characterised appear to encode a single enzyme (Chipman *et al*., 1998). The AHAS enzymes of such organisms consist of small and large subunits most similar to those of isozyme III from *E. coli*.

Eukaryotic AHAS

The catalytic subunits of eukaryotes are homologous to the large subunits of bacterial enzymes. Many plants appear to encode more than one active AHAS (Mazur *et al*., 1987; Rutledge *et al*., 1991), the functional difference of which remains to be determined. In general, multiple genes in one plant code for mature polypeptides of greater than 80% sequence homology (Chipman *et al*., 1998). At least one AHAS gene is constitutively expressed in plants (Duggleby and Pang, 2000). The highest level of expression in plants is found in actively growing tissues (Keeler *et al*., 1993; Schmitt and Singh, 1990) and more specifically the meristems (Duggleby and Pang, 2000).

Herbicide binding site

AHAS inhibiting herbicides (AHASIH) show little resemblance chemically to any of the AHAS substrates, regulators or co-factors, and act as either non-competitive or uncompetitive inhibitors. Until recently little was known about the nature and function of the herbicide binding site or the mechanism of herbicide inhibition of AHAS.

A number of bacterial, fungal and plant AHAS variants are insensitive to AHASIH. Almost all of the mutation sites in the various insensitive enzymes are highly conserved across species in wildtype AHAS but have no obvious function in catalysis or cofactor binding (Pang *et al*., 2002). Pang *et al*. (2003) provided the first view of a group B herbicide bound to AHAS and reconciled herbicide binding with known mutations that confer resistance. However the considerable structural differences between classes of group B herbicides means that interactions between the various herbicides and AHAS are likely to differ.

Regulation of AHAS

Expression of AHAS and enzymatic activity are tightly controlled and provide the key control point within the BCAA synthesis pathway (Duggleby and Pang, 2000). This is achieved through transcriptional control and feedback inhibition by the BCAAs.

2.1.3.2 AHASIH action and plant death

The precise mechanisms that link AHAS inhibition with cell death have not been elucidated (Gaston *et al*., 2002). Germination of intolerant species is not usually affected (Blair and Martin, 1988), but subsequent root and shoot growth is severely inhibited (Beyer *et al*., 1988). Although there is a cessation of BCAA synthesis (Scheel and Casida, 1985), starvation for the amino acids does not necessarily ensue, probably due to enhanced protein turnover. Build up of intermediates to toxic levels, particularly α-ketobutyrate, has been cited as a mode of action (La Rossa *et al*., 1987). A myriad of secondary effects, such as cytoplasmic acidification and the induction of fermentative metabolism, may be involved in the eventual death of intolerant plants, as explained later in this chapter.

Group B herbicides are absorbed by the roots and shoots and are transported via the xylem and phloem (Ferris *et al*., 1992). Work by Tecle *et al*. (1993) showed that over 85% of radiolabelled imazethapyr and imazaquin absorbed by soybean roots was translocated to shoots within 48 hours of treatment. Rapid metabolism immobilised the radiolabelled material. Thus the herbicide metabolites remained in the tissue where the herbicide initially accumulated, and the metabolites did not translocate to tissue that grew after treatment (Tecle *et al*., 1993).

Herbicide selectivity for plants

The selectivity of AHASIH for plants is due to the varying ability of plants to rapidly metabolise the herbicides to inactive compounds. Detoxification processes may include hydroxylation, conjugation, hydrolytic and cleavage reactions (Duggleby and Pang, 2000). Tecle *et. al.* (1993) showed that soybean first hydroxylated imazethapyr at the 5-ethyl substituent of the pyrimidine ring followed by O-glucosylation. The hydroxylated metabolite retained a reduced level of herbicidal activity compared to the parent herbicide. The glucose conjugate of the hydroxylated metabolite was completely inactive.

Enzyme resistance

The AHAS enzyme can become insensitive to group B herbicides due to mutations that can occur spontaneously in nature, be induced experimentally and selected for, or introduced via recombinant DNA techniques. There has been an effort to produce herbicide resistant crops and legumes e.g. (Heap, 2000; Howie *et al*., 2002). This is likely to lead to an increase in the use of AHASIH, increasing the urgency for an assessment on the effects of AHASIH on nitrogen fixation.

2.1.4 Persistence and degradation of AHASIH in the soil

Degradation of herbicides can occur by photolysis, chemical breakdown and microbial decomposition (Fryer and Makepeace, 1977). The availability of herbicide to microbially mediated degradation in the soil is determined by the relative rates of adsorption and desorption from soil colloids. These properties are dependent on the chemical characteristics of the herbicide, and of the soil. Environmental influences on the rate of degradation include organic matter, pH, soil moisture and temperature. The ability of a given soil bacterial population to degrade the herbicide may also vary. Insufficient or slow degradation of herbicides leads to persistence, resulting in exposure of subsequent crops and pastures to the chemical.

Degradation of sulfonylureas is dominated by chemical hydrolysis which predominates in acidic soils, and microbial breakdown which is the principle mode of degradation in

alkaline soils (Beyer *et al*., 1987). Soil pH, moisture and organic matter type and amount influence the degradation and persistence of sulfonylurea herbicides because of their influence on soil microbial activity and pH. Due to the extreme potency of sulfonylureas, undetectable levels of these herbicides may be adequate to cause injury to subsequent crops. For example residual sulfonylureas can persist into legume phase of rotations in concentrations that lead to reduced biomass production and hence N_2 fixation (Moyer *et al*., 1989).

While chemical hydrolysis can dominate the degradation of sulfonylurea herbicides, the other group B herbicides tend to be degraded by microbial activity. Microbial activity was confirmed to be responsible for the degradation of imazethapyr and imazaquin (Flint and Witt, 1997). Near complete loss of herbicidal activity was observed in non-sterile soil after 5 months whilst activity was only reduced by 14% in sterile soil. Low soil temperatures and low soil moisture limited degradation of these herbicides. 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). Microbial degradation under aerobic conditions is also the primary route of degradation for imidazolinones (Mangels, 1991).

Solution pH and organic matter are important factors influencing the adsorption of flumetsulam to soil. Murphy and Shaw (1997a) studied the soil relations of the triazolopyrimidine sulfonamide herbicide flumetsulam. Laboratory studies showed that among soils kept at constant pH, there was an inverse relationship of mobility of flumetsulam with organic matter content. The mobility of flumetsulam increased with increasing pH in soils with similar organic matter content (Shaw and Murphy, 1997a). In the field, most flumetsulam was recovered within 8cm of the surface, with the only exception being after substantial early season rainfall. Flumetsulam mobility was limited in soils with less than 1.5% organic matter content in the surface 15cm and following as much as 374 mm of precipitation. Leaching was therefore not considered a significant route of dissipation of flumetsulam in the field (Shaw and Murphy, 1997a). Persistence of flumetsulam in the field decreased with increasing accumulative rainfall and decreasing organic matter content, whilst pH had no measurable effect (Shaw and Murphy, 1997b).

The movement of imidazolinones in soil is mostly influenced by pH and organic matter, with a minor role of clay content. Binding to soil increases as pH decreases, reducing the mobility of imidazolinones. Batch equilibrium studies indicate low adsorption to soil, especially as pH increases. This is because at soil pH above the pKa of imazethapyr, the negatively charged imazethapyr molecules are repulsed from net negatively charged soil surfaces. As the soil solution pH approaches the pKa, the carboxylic acid protonates giving no net charge and reducing the repulsion between the molecule and the soil mineral surfaces. However, batch equilibrium studies have used high solution to soil ratios not indicative of field moisture levels, and have only been incubated for short periods of time. High water content slurries dilute the herbicide in solution, shifting the equilibrium to toward lower adsorption. Thus mobility and leaching potential of herbicides in unsaturated soil is overestimated. Johnson *et. al.* (2000) studied the adsorption of imazethapyr on a sandy loam over a range of pHs and at 16% moisture for 30 days. There was a rapid adsorption of imazethapyr to the soil in the first day of incubation at all pHs. The concentration of imazethapyr in the soil water continued to decline slowly thereafter due to continued adsorption. Soil column mobility studies also showed a rapid decline in mobility as time increases (Johnson *et al*., 2000).

It is likely that group B herbicides persist and accumulate in the soil, especially in alkaline soils and low rainfall areas. This increases the potential for damage to legume crops and pastures.

2.2 Nitrogen fixation by legumes

Legumes are unique in that they can obtain combined nitrogen from their symbiosis with rhizobia. Should this symbiosis be compromised, legumes are capable of assimilating soil nitrogen. Since reductions in symbiotic nitrogen fixation may not be obvious and are difficult to assess in the field, the impact of herbicides on nitrogen fixation is rarely assessed. Therefore there is a dearth of knowledge in relation to the impact AHASIS have on the contribution of legume N to farming systems.

2.2.1 Symbiotic N_2 fixation

The symbiosis between legumes and rhizobia is a very complex phenomenon. This symbiosis has been studied for over a century and the extensive literature on this topic makes a comprehensive review here inappropriate. However, an outline of the processes involved follows, with due reference to the literature made in discussing the specific effects of AHASIH on particular aspects where required. A general outline of nodulation is given by Gordon *et al*. (2001) and Schultze and Kondorosi (1998) reviewed the regulation of nodule development. At least 100 genes from each partner must interact in a favourable environment for symbiotic nitrogen fixation to occur (Vance and Lamb, 2001). An analysis of nodulation from a molecular genetic point of view is reviewed by Rolfe *et al*. (1995), as well as Broughton and Perret (1999), Luyten and Vanderleyden (2000) and Pawlowski (1997). Sprent and Sprent (1990) adapted a framework of nodulation by Vincent (1980) to provide an overview of the stages involved in a typical root hair infection system. The following description is based on that framework.

Multiplication of rhizobia on (or near) the root surface: colonization

Rhizobia survive in the soil as saprophytes. Colonisation of the rhizosphere can occur via chemotaxis and/or electrotaxis then subsequent multiplication is stimulated by various compounds such as glycans, carboxylic acids, amino acids and flavonoids in the root exudates of legumes.

A specific recognition between the legume and rhizobia is required for the initiation of the symbiosis, leading to the development of the nodule, and the invasion of plant roots by rhizobia. Two classes of genes are involved. The first set is for the synthesis, assembly and transport of bacterial cell surface components such as cyclic β-glucans, exopolysacharides, lipopolysaccharides and capsular polysaccharides. The second set are the nodulation genes, which play and important role in signal exchange. Flavonoids produced by the host plant induce the expression of bacterial nodulation genes which regulate the synthesis of lipochitooligosacharides, the Nod factors. Nod factors induce morphological and electrophysiological changes in the roots and induce the expression of early nodulin genes required for nodule formation.

Figure 2.7 Nodulation (Richardson *et al*., 1989)

Adhesion of rhizobia to the root hair surface

Attachment to root hairs occurs in stages with increasing levels of specificity. Plant lectins and bacterial polysaccharides are involved (Sprent and Sprent, 1990). Root cells are only transiently susceptible to nodulation by rhizobia (Bhuvaneswari *et al*., 1981).

Root hair curling

Root hair curling is a prerequisite for infection in many agricultural legumes (infection of lupins is by intercellular entry (Gonzalez-Sama *et al*., 2004)), and is dependent on the physiological conditions and surface polysaccharides of the rhizobia (Sprent and Sprent, 1990).

Formation of infection threads (in root hairs)

The root hair wall is breached within the curl of the root hair, and the infection thread is formed. The infection thread consists of newly synthesized host cell wall material surrounding the bacteria. This structure is surrounded by a host membrane continuous with the root hair plasmalemma (Sprent and Sprent, 1990).

Nodule initiation: formation of nodule meristem, nodule development and differentiation

Cell division in the root cortex may be stimulated by Nod factors before infection takes place, eventually forming the nodule meristem. Two types of nodules exist (Figure 2.8). Determinate nodules do not have a persistent meristem, so they develop to a

certain size and cannot grow further. Indeterminate nodules may continue to grow away from their point of attachment to the root. Distinct zones of cells range from small rapidly dividing meristematic cells at the tip of the nodule, newly infected cells, mature cells containing active bacteroids to senescent cells closest to the root attachment (Gordon *et al*., 2001). The type of nodule formed depends on the host.

Figure 2.8: Structure of determinate and indeterminate nodules (Hansen, 1994)

Bacteroid release of rhizobia from infection threads and differentiation

Bacteria are 'released' from the infection thread and differentiate into bacteroids, but always remain bounded by the peribacteroid membrane and therefore distinct from the host.

Onset of nitrogen fixation

Nitrogenase synthesis normally follows shortly after release of bacteria from infection threads. Leghaemoglobin is produced in the host cells for the maintenance of high

oxygen flux to satisfy respiration requirements at low concentration so as not to inhibit the oxygen sensitive nitrogenase enzyme.

Biochemical and physiological (complementary) functions associated with nitrogen fixation

The metabolism of the two symbionts is complementary. Bacteroidins such as nitrogenase are produced by bacteroids and the host cells produce nodulins such as leghaemoglobin. Plants provide energy and nutrients and in return approximately 90% of the fixed nitrogen is rapidly translocated from the endophyte to the host.

Maintenance (persistence) of nodule function

Bacteroid division occurs after nitrogen fixation has begun. A nodule vascular system is essential for transport of metabolites and solutes, and water to transport fixation products. Leghaemoglobin, and variable resistance to diffusion maintain gaseous exchange of oxygen across the peribacteroid membrane.

Nodule function and senescence is influenced by the environment. A change in colour from pink to greenish brown may be observed as nodules senesce, but this may be partly reversible.

There are a large number of biologically diverse requirements for successful symbioses between legumes and rhizobia to form and function. Each of these requirements presents a potential way in which group B herbicides can affect nitrogen fixation.

2.3 AHAS inhibiting herbicides and nitrogen fixation

There are four aspects that provide a useful framework with which to consider the possible impacts of AHASIH on symbiotic nitrogen fixation. Herbicides can impact upon either (1) the legume symbiont; or (2) the rhizobia symbiont; or (3) the interactions between the symbionts; or (4) the nitrogen fixation process itself (Eberbach, 1993). Each of these are discussed below. In addition, extraneous factors or stressors that ordinarily impact on nitrogen fixation may be exacerbated by AHASIH.

2.3.1 Effects on nitrogen fixation

Potential impacts of AHASIH on nitrogen fixation are often masked by adequate levels of soil N and other factors that constrain growth and yield. Thus the total nitrogen content of herbicide treated plants may appear to be unaffected when in fact plant nitrogenase activity could be reduced as a result of interference by herbicides. Therefore special attention must be given to the possible effects of herbicides on nitrogen fixation by legumes.

Sawicka *et al*. (1996) observed a decrease in nitrogenase activity measured by acetylene reduction assays in peas, lupins and soybeans due to imazethapyr. Reduced nitrogen fixation assessed by acetylene reduction was also evident in all alfalfa and red clover nodules grown in chlorsulfuron-treated soil (Martensson and Nilsson, 1989).

The specific nitrogen fixation rate in pea nodules as measured by acetylene reduction activity at the end of a four week study by Royuela *et. al.* (2000) was reduced by imazethapyr. There was an impairment of whole plant nitrogen fixation due to the decrease in specific nitrogen fixation and reduced nodule biomass. However plant organic nitrogen content expressed as a percentage of dry weight only showed a slight decline after imazethapyr treatment. Plants did not appear to be under nitrogen shortage, since one of the primary symptoms of N deficiency, chlorosis in mature leaves, was absent. Chlorosis was observed only in the youngest leaves which is not a symptom of N deficiency. One explanation is that a decrease in growth and nitrogen demand occurs concomitantly. The inhibition of BCAA biosynthesis and subsequent increase in free amino acid levels may elicit a decrease in nitrogen demand and hence fixation through existing regulatory mechanisms.

In context of the conceptual framework suggested by Eberbach (1993), the effects of a herbicide on nitrogen fixation refer to more direct effects than the other three areas. According to a strict definition of ‘direct’, effects on nitrogen fixation means that the nitrogenase enzyme itself is inhibited by the herbicide. While inhibition of nitrogenase resulting from herbicide application has been measured, no herbicide is known to have the direct inhibition of nitrogenase as its mode of action and therefore any impact of a herbicide on nitrogen fixation itself must be indirect. Therefore any effects of

herbicides on nitrogen fixation are likely to be via the other three pathways; namely the legume symbiont, the rhizobia symbiont, or interactions between the two.

2.3.2 Effects on the legume

2.3.2.1 Growth

The amount of biomass produced is important where legumes are used as feed and also because incorporation into soil (for example green manuring) can contribute to soil N status. The impact of AHASIH on nitrogen fixation can be as simple as a reduction in legume biomass, since the amount of N_2 fixed per hectare is a product of biomass per hectare and N concentration of that biomass less any nitrogen assimilated from the soil. It must also be recognised that even if yield and biomass are not reduced, nitrogen fixation may still be affected. The growth of legumes in which nitrogen fixation is not optimal is likely to use more soil N, resulting in a less positive and potentially negative effect on soil N status where N is removed in harvested products (e.g. grain, meat etc.). The literature on the effect of AHASIH on legume growth is described below.

Many crop and pasture legumes (soybeans, field pea, lentils, dry bean, faba bean, lucerne) are considered to have good general tolerance to pre and early post-emergence applications of imidazolinone herbicides (Hart *et al*., 1991). However, some negative effects on the growth of legumes treated with either imazethapyr or sulfonylurea herbicides have been reported. These are summarised in Table 2.3.

Laboratory experiments show the potential for imazethapyr to significantly reduce shoot and root growth of plants (Gonzalez *et al*., 1996; Royuela *et al*., 2000). The herbicide's effects in the field appear to be more variable as they are dependent on edaphic and climatic conditions. Under field conditions imazethapyr was shown to induce early symptoms such as internode shortening, stunting, and leaf crinkling in several legume species (Table 2.3). In addition some legumes exhibited delayed maturity (bean, lentil, pea) and reduced biomass production (chickpea, *Medicago*, soybean, *Trifolium* sp.). Although it was shown that imazethapyr does not affect seed yield, a reduction in

biomass is sometimes seen, indicating the potential for imazethapyr to affect nitrogen fixation.

Even though sulfonylureas are not recommended for in-crop usage in legumes, residual sulfonylurea can persist in the soil into a legume phase of a rotation, particularly on alkaline soils and under low soil moisture conditions that slow degradation (see section 2.1.4).

Several studies have shown the effects of very low levels or residual levels of sulfonylurea herbicides on the legumes (Table 2.3). For example, chlorsulfuron applied at 2 g ha^{-1} did not affect emergence of lucerne (alfalfa), however subsequent growth was strongly inhibited (Martensson and Nilsson, 1989). These plants recovered after 5-6 weeks, however no such recovery was observed at 4 and 8 g of chlorsulfuron per hectare. In laboratory experiments the base of root hairs of uninoculated lucerne became swollen at 0.28 ρM chlorsulfuron, a likely level to persist in the year after application. This was thought to inhibit early root hair infection. Inoculated plants showed atypical root deformations (Martensson and Nilsson, 1989). The effect of chlorsulfuron on lucerne was greater in limed treatments providing evidence that inhibition is greater at higher pH (Koopman *et al*., 1995). Gillet and Holloway (1996b) showed very low levels of triasulfuron (1% and 4% of the original application rate, to represent the estimated residual range in the topsoil) in alkaline soils significantly reduced biomass production of annual medic shoots, and severely restricted root penetration.

The effects of chlorsulfuron on nitrogen fixation in a field experiment were examined by Anderson (2001). Chlorsulfuron at 1.5, and 3 g a.i. ha^{-1} (the recommended rate is 15 g a.i. ha^{-1}) reduced shoot dry weight of chickpea by 36% and 49% respectively and reduced nitrogen fixation (kg N_2 fixed ha^{-1}) by 40% and 57% respectively. Thus there was a greater reduction in the amount of nitrogen fixed than that of shoot dry weight.

Table 2.3 Effects of imazethapyr, flumetsulam, chlorsulfuron, triasulfuron and metsulfuron methyl on pasture and grain legumes

Herbicide/ Plant	Conditions	Rate	Observed effects	Reference
Imazethapyr				
Alfalfa (*Medicago sativa*)	Field	Not specified. Recommended rate?	Early internode shortening. Intolerant to pre-plant soil applications	Hart *et al.* (1991)
Bean (Dry) (*Phaseolus vulgaris*)	Field	Not specified. Recommended rate?	Delayed maturity	Hart *et al.* (1991)
	Field (United States)	70-100 g ha^{-1}	Stunting, Leaf crinkling, interveinal chlorosis, reduced height , delayed maturity but yields unaffected	Wilson and Miller (1991)
Chickpea (Cicer arietinum)	Field	72 g a.i. ha^{-1}		
	Field	29 g a.i. ha^{-1}	Shoot dry weight and N2 fixation reduced by 52%	Anderson 2001
	Field (Australia - Qld)	72 g a.i. ha^{-1}	Unaffected	Barnes *et al*. (1996)
Lentils (*Lens culinaris*)	Field	Not specified. Recommended rate?	Delayed maturity	Hart *et al.* (1991)
Medic (Annual) (*Medicago truncatula*)	Field	48 g a.i ha^{-1}	Seed yield unaffected	Dickinson *et al*. (1993)
	Field (Australia - SA)	48 g a.i ha^{-1}	Reduced shoot dry weight	Fajri *et al.* (1996)
Pea (Field) (*Pisum sativum*)	Field	Not specified. Recommended rate?	Delayed maturity	Hart *et al.* (1991)
	Field (Australia - SA)	Not specified. Recommended rate?	Safe	Wheeler and Jeffries (1996)

	Laboratory	>1.73uM	Inhibited height, reduced shoot dry weight, root dry weight	Gonzalez *et. al*. (1996)
	Laboratory	0.7 mg L^{-1}	Shoot dry weight reduced by 70% and root dry weight reduced by 80%	Royuela *et al.* (2000)
Pea (Dry) (*Pisum arvense*)	Field	Not specified. Recommended rate?	Delayed maturity	Hart *et al.* (1991)
Cowpea			Tolerant	Baerg and Barret (1996)
Soybean (*Glycine max)*	Field	>70 g a.i. ha^{-1}	Internode shortening under stressful conditions, chlorosis, crinkling of upper leaves.	Hart *et al.* (1991)
	Field	35 g a.i. ha^{-1}	Reduced shoot dry weight	Scarponi *et al.* (1995)
Subterranean clover (*Trifolium sp*)	Field (Australia - SA)	48 g a.i ha^{-1}	Reduced shoot dry weight	Fajri *et al.* (1996)

Flumetsulam

Pea (Field) (*Pisum sativum*)	Field (Australia - SA)	25 and 50 g ha^{-1}	Safe	Wheeler and Jeffries (1996)
Lentils (*Lens culinaris*)	Field (Australia - SA)	25 and 50 g ha^{-1}	Safe	Wheeler and Jeffries (1996)
Chickpea *(Cicer arietinum)*	Field (Australia - SA)	25 and 50 g ha^{-1}	Tolerant	Wheeler and Jeffries (1996)
	Field (Australia - Qld)	20 g a.i. ha^{-1}	Unaffected	Barnes *et al*. (1996)
Subterranean clover (*Trifolium sp.*)	Field (Australia)	25 g ha^{-1}	Tolerant	Gilmour (1996)
	Field (Australia - SA)	25 g ha^{-1}	Reduced shoot dry weight	Fajri *et al.* (1996)

Medic (Annual) (*Medicago truncatula*)	Field (Australia - SA)	20 g a.i ha^{-1}	Seed yield unaffected	Dickinson *et al*. (1993)

Chlorsulfuron

Alfalfa (*Medicago sativa*)	Field	2 g ha^{-1}	No effect on emergence but subsequent growth reduced	Martensson and Nilsson (1989)
	Laboratory	0.28pM	Root hair swelling of uninoculated roots and atypical root deformations of inoculated plants.	Martensson and Nilsson (1989)
		Residual 275 days	Growth inhibition	Brewster and Appleby (1983)
	Field (Canada)	Residual 6 years	Recovery after this time	Moyer *et al*. (1990)
	Pot	5 g ha^{-1}	Reduced shoot regrowth, effect more severe at higher pH.	Koopman *et al.* (1995)
Medicago rugosa	Field	Residual	Reduced shoot dry weight (31-60%)	Anderson 2001
Chickpea *(Cicer arietinum)*	Field	0.75-3 g a.i. ha^{-1}	reduced shoot dry weight (22-49%), Reduced N_2 fixation (40-57%)	Anderson 2001
	Soil free laboratory system		ID50=1.01 *µg* a.i. L^{-1}	Churchett *et al*. (1996)
		Residual 12 months	Shoot tip yellowing, reduced root growth and yield, premature death	Ferris *et al*. (1992)
	Greece	Residual 8-14 months	Reduced plant density, dry weight, grain yield	Efthimiadis *et al*. (1989)
Clover		Residual 4 months	Seedlings affected	Eberbach and Douglas (1991)
Medic (Annual) (*Medicago truncatula*)	Field	<0.02 g a.i. ha^{-1}	Reduced shoot dry weight, root dry weight, nodulation	Rovira *et al.* (1993)
	Field	Residual 12 months	Purpling, yellowing, stunting, reduced plant numbers	Evans *et al.* (1993)

Soybeans	Field	Residual 12 months	Significant injury	Petersen and Arnold (1985)
	Field	Residual 24 months	No injury	Petersen and Arnold (1985)
	Field (United States)	Residual 12 months	36 g ha^{-1} reduced yield after 12 months	Ritter *et al* (1988)
Pea (Field) (*Pisum sativum*)	Field (Manitoba Canada)	Residual 12 months	Visible injury	Friesen and Wall (1991); Moyer *et al*. (1990)
	Greece	Residual 8-14 months	Reduced plant density, dry weight, grain yield	Efthimiadis *et al*. (1989)
Lentils (*Lens culinaris*)	Field (Manitoba Canada)	Residual up to 3 years	22 g a.i. ha^{-1} caused injury	Friesen and Wall (1991); Moyer *et al*. (1990)
	Canada	Residual up to 7 years	Injury observed	Moyer *et al*. (1990)
	Greece	Residual 8-14 months	Reduced plant density, dry weight, grain yield	Efthimiadis *et al*. (1989)
	Greece	Residual	Significant injury up to 8 months	Kotoula-Syka *et al*. (1993)
Bean	Field (Canada)	Residual 4 years	Recovery after this time	Moyer *et al*. (1990)

Triasulfuron

Medic (Annual) (*Medicago truncatula*)	Field (Australia - SA)	0.01-0.04 x RR	Reduced shoot dry weight and root penetration in >7 pH soils.	Gillett and Holloway (1996a)
	Field	Residual 12 months	Reduced shoot dry weight and seed yield	Evans *et al.* (1993)
Pea (Field) (*Pisum sativum*)	Greece	Residual 8-14 months	Tolerant	Efthimiadis *et al*. (1989)
Lentils (*Lens culinaris*)	Greece	Residual	Significant injury up to 8 months	Kotoula-Syka *et al*. (1993)
	Greece	Residual 8-14 months	Tolerant	Efthimiadis *et al*. (1989)
Chickpea *(Cicer arietinum)*	Laboratory		ID50=1.27 μg a.i. L^{-1}	Churchett *et al*. (1996)

	Greece	Residual 8-14 months	Tolerant	Efthimiadis *et al*. (1989)
Sub Clover (*Trifolium subterraneum*)	Laboratory	Residual	Lethal	Eberbach and Douglas (1991)

Metsulfuron methyl

Lentils (*Lens culinaris*)	Greece	Residual	Injury up to 4 months but not at 8 months	Kotoula-Syka *et al*. (1993)
Chickpea *(Cicer arietinum)*	Laboratory		ID50=3.73 µg a.i. L^{-1}	Churchett *et al*. (1996)
Medic (Annual) (*Medicago truncatula*)	Field (Australia - SA)	Residual 12 months	4 g a.i. ha^{-1} reduced shoot dry weight and seed yield	Evans *et al.* (1993)

2.3.2.2 BCAA synthesis and nitrogen metabolism

Group B herbicides inhibit branched chain amino acid biosynthesis by inhibiting the AHAS enzyme. Disruption of this fundamental metabolic process is likely to have secondary effects because of the requirement for these amino acids in protein (including enzymes) synthesis. It is therefore feasible that in legumes a reduction in BCAA synthesis could result in reduced levels of nitrogen fixation.

A reduction in BCAA synthesis due to the inhibition of the AHAS enzyme by group B herbicides can result in an increase amino acid turnover to compensate for the decreased availability of valine, leucine and isoleucine. For example, Rhodes *et al*. (1987) studied the influence of chlorsulfuron on amino acid metabolism of duckweed (*Lemna minor* L.). There was an increase in total free amino acid levels within one day of treatment. This was more likely due to increased protein turnover than new amino acid synthesis. As the concentration of chlorsulfuron was increased, valine, leucine and isoleucine became a smaller proportion of the free amino acid pool. ^{15}N tracer studies revealed that chlorsulfuron inhibited the incorporation of ^{15}N into valine, leucine and isoleucine,

consistent with the cessation of synthesis of these amino acids. Similar results were found by Scarponi *et al*. (1995) following the application of imazethapyr at 35g a.i. ha^{-1} to soybean shoots. Although the total free amino acid pool did not change significantly, the amounts of valine, leucine and isoleucine in shoots of soybean decreased with respect to untreated controls. Imazethapyr also caused a decrease in soluble protein levels and an increase in the free amino acid pool in pea plants (Gaston *et al*., 2002; Royuela *et al*., 2000); however, only the valine content declined, while isoleucine and leucine increased.

A reduction in BCAA cycling can result in an accumulation of precursor compounds such as α-ketobutyrate (Figure 2.6). Royuela *et al*. (2000) observed an increase in threonine (the precursor of α-ketobutyrate, a substrate of AHAS (Figure 2.6)) and α-aminobutyric acid (the transamination product of α-ketobutyrate) following application of imazethapyr to peas. The main contributors to the increase in free amino acid pool were those derived from TCA cycle intermediates (asparagine and glutamine) and three glutamate derivatives (ornithine, proline and γ-aminobutyric acid). Similar results were observed by Rhodes *et al*. (1987) for duckweed treated with chlorsulfuron, and Hofgen *et al*. (1995) using a potato transformed with a constitutively expressed antisense construct of the AHAS gene. The transgenic potato plants had reduced AHAS gene expression which resulted in severe growth retardation and phenotypic effects which mimicked the effects of applied AHAS inhibiting herbicide on potato plants. Steady state levels of AHAS mRNA were drastically but not completely reduced in the transgenic plants, leading to a significant reduction in AHAS activity. As would be expected, chemical inhibition of AHAS (using imazaquin) in wild-type plants did not affect expression levels of the AHAS gene. Amino acid levels increased in transgenic and herbicide treated wild-type plants, with the exception of a reduction of valine in sink tissues of herbicide treated plants. Unlike in Rhodes *et al*. (1987) an accumulation of α-ketobutyrate was not detected in AHAS antisense or herbicide treated plants under normal physiological conditions. However, accumulation in antisense and herbicide treated plants but not controls occurred when threonine (precursor to α-ketobutyrate) was fed to isolated leaves. This accumulation could be due to a reduced capacity to metabolise α-ketobutyrate (Hofgen *et al*., 1995).

Scarponi *et. al.* (1997) observed changes to protein formation and the free amino acid profile in faba bean treated with chlorimuron-ethyl (a sulfonylurea herbicide used in soybeans), imazethapyr and propachlor. Valine, leucine, isoleucine and total N were decreased by chlorimuron-ethyl, while total soluble N was increased. Inhibition of glutamine synthetase (associated with ammonia assimilation) followed the inhibition of AHAS, but preceded that of nitrite reductase (associated with ammonia production). Earlier work by Scarponi *et al*. (1995) showed that imazethapyr treatment of soybean did not immediately reduce the activity of nitrate reductase, but a decline in nitrite reductase was observed after 24 hours, indicating potential starvation for ammonia. Glutamine synthetase and glutamate synthase activity were also depressed by treatment with imazethapyr. Recoveries in the activities of these enzymes were observed after 24 hours when valine, leucine and isoleucine were included in the imazethapyr treatment. Due to this delay the reduction in these activities seemed to be a response to the lack of ammonia rather than to direct consequences of AHAS inhibition (Scarponi *et al*., 1995). The hypothesis was put forward that in soybean shoots treated with imazethapyr, the demand for ammonia in connection with decreased synthesis of branched chain amino acids led to the acceleration of protein turnover (Scarponi *et al*., 1995).

The perturbations to nitrogen metabolism and particularly amino acid cycling in plants caused by group B herbicides are likely to have implications for nitrogen fixation. It remains to be determined how direct the link between the inhibition of BCAA synthesis and nitrogen fixation is.

2.3.2.3 Other plant responses

Many secondary responses to AHASIH have been postulated and observed. Examples include changes to carbon metabolism, production of secondary plant metabolites and aerobic fermentation associated with stress responses, altered electron transport and ultrastructural changes to meristematic tissues. Each of these are discussed below and may impact on nitrogen fixation in ways yet to be considered.

Scarponi *et al*. (1996) observed a decrease in starch content of soybean roots and shoots 36 hours after treatment with imazethapyr. There was an increase in glucose content in shoots until 60 hours after imazethapyr treatment. After an initial increase, the glucose

content of roots declined. This was thought to be due to less transport of soluble carbohydrates following imazethapyr treatment. An observed decrease in RUBISCO activity (CO_2 assimilation) was consistent with lower CO_2 fixation via feedback due to an inhibition of transport of soluble carbohydrates. Addition of valine, leucine and isoleucine prevented the changes in glucose and starch levels, and RUBISCO activity (Scarponi *et al*., 1996).

The accumulation of total soluble sugars and starch in both the leaves and roots supports the hypothesis that carbohydrate accumulation in leaves results from an impaired root metabolic activity and therefore a decrease in root sink strength. Sucrose synthase and alkaline invertase activity were reduced, indicating that imazethapyr impaired the ability to hydrolyse sucrose (Gaston *et al*., 2002). Royuela *et. al.* (2000) showed that long term imazethapyr treatment did not impair the capacity to synthesise carbohydrate, as there was a negligible effect on photosynthesis. The accumulation of carbohydrates (sucrose and starch) in leaves suggested that these carbohydrates were not being used for growth, maintenance and translocation. Roots also showed an accumulation of carbohydrates as soluble sugars and starch. No significant changes in carbohydrate levels were detected in nodules either, which suggests that sucrose availability for nodules was not limiting for nitrogen fixation (Royuela *et al*., 2000).

Increases in phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) activities were observed after treating soybean with imazethapyr. These are key enzymes in the biosynthesis of phytoalexins, which are produced as a natural response to biotic and abiotic infections. Addition of valine, leucine and isoleucine did not prevent these changes, which therefore are not a direct consequence of the inhibition of AHAS (Scarponi *et al*., 1996).

Gaston *et al*. (2002) observed a transient increase in pyruvate in pea roots after 1 day of imazethapyr treatment. Activities of pyruvate decarboxylase, lactate dehydrogenase and alanine amino transferase increased in roots. An increase in the activity of alcohol dehydrogenase, which uses the product of pyruvate decarboxylase as a substrate was also observed. An increase in the activity of these enzymes indicated the utilisation of pyruvate by other metabolic pathways due to the transient increase in substrate availability in the absence of AHAS activity. Alternatively, these activities could have

been enhanced by an inhibition of mitochondrial respiration or by a shortage of oxygen caused by imazethapyr. A 17% inhibition of aerobic respiration (as measured by oxygen uptake) was observed after 3 days and up to 39% after 7 days of treatment. Therefore aerobic fermentation was elicited after 1 day of imazethapyr treatment, but was is not triggered by the inhibition of root respiration (Gaston *et al*., 2002).

Fermentation has a general function in aerobic metabolism under stress conditions. The induction of fermentative pathways in pea plants by the inhibition of AHAS may have physiological consequences in addition to the lack of BCAA biosynthesis. Fermentative activities produce acetaldehyde, ethanol and lactate. These chemicals may be toxic to plants, leading to a slow death even though aerobic conditions exist.

Electron transport for the establishment of a proton gradient in plant mitochondria can occur through the cytochrome pathway, or the cyanide resistant respiratory pathway catalysed by the alternative oxidase (AOX) (Day *et al*., 1980). Electron flow through the cytochrome chain generates a proton gradient from complexes I, III and IV and the potential energy is used to synthesize ATP via complex V. When electrons flow through AOX, only complex I can contribute to the proton gradient, and the energy flow that is not used to generate ATP is dissipated as heat. AOX has a role in thermogenic plants to volatize odiferous compounds that attract pollinators using the heat generated. In non-thermogenic plants, the role of AOX remains unclear, but activity has been shown to be influenced by environmental, developmental, chemical and tissue specific signals. Aubert *et al*. (1997) found that sulfometuron methyl, chlorsulfuron and imazaquin triggered a dramatic induction of the alternative oxidase in sycamore suspension cells, and a corresponding decrease in cytochrome pathway activity. The metabolic perturbations induced by the herbicides, namely arrest of branched chain amino acid biosynthesis, were responsible for these effects (Aubert *et al*., 1997). The possible implications of this to nitrogen fixation are yet to be considered.

Ultrastructural changes in meristematic root cells and leaf mesophyll cells were observed after treatment of pea plants with chlorsulfuron (Stoynova *et al*., 1997). Mitochondria in treated plants had a visibly translucent matrix and a reduced number of cristae and ribosomes. Structural disturbances in chloroplasts were similar to those observed during senescence. Alterations in the ultrastructural organization of the

nucleus included a reduction in volume, and a segregation of its fibrillar and granular components. The nuclei, mitochondrion and chloroplast ultrastructure showed a decrease of transcriptional and translational activity. These changes were consistent with the synthesis of toxic BCAA intermediates and could be observed long before changes due to amino acid starvation. The lack of distinct changes in the ribosome density in the cytoplasm indicated that the reduction in the bulk of the cell proteins occurs first in the organelles, especially the chloroplasts, but not the cytoplasm. The exchange of energy and metabolites between organelles appeared to be inhibited. The formation of chloroplast protrusions, swelling of thylakoids, changes in starch grains and plastaglobules after treatment with chlorsulfuron were non-specific symptoms of stress, and can be regarded as signs of premature senescence (Stoynova *et al*., 1997).

Ultimately all proteins and enzymes are made up of amino acids including the BCAAs produced by the pathway inhibited by group B herbicides.

2.3.3 Effects of herbicides on rhizobia

Most studies on the effects of herbicides on legume growth, nodulation and nitrogen fixation conclude that herbicide induced reduction of nodulation or nodule activity is likely to be due to adverse effects of the herbicide on the plant. This is because herbicides at concentrations used in the field are unlikely to affect growth and survival of rhizobia. However the possibility that the exposure of rhizobia to herbicides may alter their ability to infect the host plant has not been thoroughly studied. It is possible that the ability of rhizobia to infect is inhibited independent of effects on the growth of rhizobia. Repeated or continuous exposure to herbicides may alter physiological responses of bacteria without manifesting in a reduction of growth.

2.3.3.1 Growth and survival of rhizobia

The size of the population of rhizobia influences nodule formation. High rhizobia density in the rhizosphere promotes early infection and nodulation (Lim, 1963). It is therefore feasible that should AHASIH impact upon the growth and survival of rhizobia, nodulation will be affected. However most studies conclude that the growth of rhizobia is not affected by AHASIH.

The growth of rhizobia was not inhibited in both complex and defined media by concentrations of commercial or technical grade imazethapyr from 0.34 mM to 3.4 mM representing a range in excess of field rates of application of herbicide (Gonzalez *et al*., 1996). In a field study, the number of bacteria in soil under legume crops was not adversely affected by imazethapyr. Imazethapyr seemed to stimulate development of bacteria, with an increase in the total number of bacteria compared to untreated plots (Sawicka *et al*., 1996).

Growth of rhizobia was not affected by chlorsulfuron at 0.55 and 5.5 μM corresponding to approximately 50 and 500 times the recommended field application rate of 4 g ha^{-1} (Martensson and Nilsson, 1989).

Growth of chickpea inoculant strain CC1192 in yeast mannitol broth was not affected by chlorsulfuron, imazethapyr and flumetsulam at double the recommended application rate (Anderson, 2001). Similar results were obtained when rhizobial cultures were grown in a defined media free of amino acids, in the presence of chlorsulfuron (Anderson, 2001).

2.3.3.2 Rhizobial AHAS

When AHAS was extracted from *Rhizobium leguminosarum* biovar. *viciae*, *in vitro* assays showed that the specific activity of isolated rhizobia AHAS was nearly 20 times the activity of AHAS extracted from pea (*Pisum sativum*) leaves (Royuela *et al*., 1998). Bacteroids from nodules also had high AHAS specific activity. The plant fraction of nodules had higher activity than other plant tissues. AHAS activity of free-living rhizobia and bacteroids was only slightly less sensitive to imazethapyr than that of pea tissues (Royuela *et al*., 1998). The magnitude of the bacterial enzyme's resistance compared with that of plants was not sufficient to explain the growth tolerance of rhizobia to AHAS herbicides. It was proposed that the tolerance of rhizobia to imazethapyr was explained mostly by higher specific activity of the AHAS, and only marginally by the enzyme being less sensitive to the herbicide.

The response of microorganisms to different AHASIH varies. Burnet and Hodgson (1991) studied the effects of sulfometuron methyl and chlorsulfuron on the growth and AHAS activity of a variety of microorganisms. Sulfometuron methyl was a more potent inhibitor of bacterial growth than chlorsulfuron, possibly because it can move more readily through the cell membrane. Effects of an AHASIH on microorganisms may depend on which AHAS isozyme/s the microorganism possesses and the ability of the microorganism to degrade particular herbicides. For example, Burnet and Hodgson (1991) have shown that *Aspregillus niger* can degrade chlorsulfuron. It is likely that sulfonylureas will change the composition of the microbial community in soils due to the differential effects they have on microorganisms.

Forlani *et. al.* (1995) also found differential sensitivity among plant-associated bacteria to chlorsulfuron, rimsulfuron, imazapyr and imazethapyr. On the whole, growth inhibition was related to the sensitivity of each strain's acetohydroxyacid enzyme to the herbicides. This varied greatly among strains belonging to the same genus, or even species. The growth of more than 75% of strains tested were unaffected by the herbicides at or below concentrations approximating the highest field application rate. Two out of 18 rhizobacterial strains were inhibited by the sulfonylureas, with rimsulfuron more potent than chlorsulfuron. Two other strains were inhibited by the imidazolinones, with imazapyr more potent than imazethapyr. Shifts in microbial community structure were demonstrated. The implications for colonisation of plants were demonstrated when the presence of rimsulfuron in the soil promoted root occupancy of a resistant strain when it was co-inoculated with a sensitive strain (Forlani *et al*., 1995).

A greater tolerance of symbiotic pea plants to imazethapyr than non-nodulated plants has been observed (Gonzalez *et al*., 1996). Nodulated plants may have an additional source of AHAS and hence branched-chain amino acids due to the high AHAS activity in bacteroids (Royuela *et al*., 1998). The contribution of bacteroids to amino acid biosynthesis in plants has not been comprehensively studied. Unpublished results by Royuela show that *in vivo* application of imazethapyr to nodulated pea plants does not produce a decrease in the pool of branched-chain amino acids in nodules.

2.3.3.3 Other physiological effects of AHAS inhibition in bacteria

La Rossa *et. al.* (1987) suggested that accumulation of α-ketobutyrate to toxic levels was caused by the inhibition of AHAS in *Salmonella typhimurium*. A large number of Tn*10* insertion mutants that were hypersensitive to sulfometuron methyl displayed defects in the catabolism of α-ketobutyrate. α-ketobutyrate toxicity was therefore cited as an important contributor to the herbicidal action of AHASIH (La Rossa *et al*., 1987).

Van Dyk *et. al.* (1998) used a reporter construct in *E. coli* to identify promoters activated when cells were exposed to sublethal doses of sulfometuron methyl (to cause 25% inhibition of growth). 12 sulfometuron methyl induced fusions were identified. Basal levels of expression of six of the fusions were reduced by inactivation of the σ^s sigma factor, indicating that they are under the genetic control of this regulon. The weak acid salicylate, which causes cytoplasmic acidification, increased the expression of seven of the fusions. No promoters associated with the amino acid starvation response were identified, indicating that isoleucine and valine starvation were not severe. This was consistent with the lack of induction of the heat-shock controlled *grpE* promoter, which normally responds to amino acid starvation. Sulfometuron methyl induction of the fusions was lost in the presence of isoleucine and valine. So although the induced promoters were clearly linked to sulfometuron methyl inhibition of branched chain amino acid biosynthesis, the responses were due to more subtle physiological perturbations since starvation for these amino acids was not severe (Van Dyk *et al*., 1998). It remains to be seen whether such effects of herbicides on the physiology of bacteria may affect nodulation and nitrogen fixation.

2.3.4 Effects on nodulation

The inhibition of nodulation is generally attributed to a disruption of the early events of nodulation such as stimulation of root hair curling and formation of infection threads (McKay and Djordjevic, 1993). This can occur even when the growth of either symbiont is not affected. Poor nodulation has been associated with a variety of conditions in the field such as unfavourable pH, high salinity, toxicity of elements such as aluminium, high nitrate, temperature extremes and deficiencies of essential elements such as calcium and phosphorus. AHASIH may exacerbate or interact with these factors, or impede nodulation directly.

AHAS inhibiting herbicides and nodulation (number and mass)

Reduced nodule number on legumes following exposure to AHASIH is well documented for lucerne (Koopman *et al*., 1995); annual medic (Rovira *et al*., 1993); pea (Gonzalez *et al*., 1996; Royuela *et al*., 2000); and chickpea (Anderson, 2001). Examples of reduced nodulation due to group B herbicides and proposed mechanisms are discussed below.

Gonzalez *et. al.* (1996) showed that treatment of pea plants with 1.73 μM imazethapyr reduced the numbers of nodules per plant by 45% despite growth of pea plants not being affected at this concentration. The size of nodules was not affected, suggesting an effect on nodule initiation rather than on nodule development. At doses higher than 1.73 μM nodule formation was nearly eliminated (Gonzalez *et al*., 1996). Inhibition of nodule formation was thought to be related more to the toxicity of imazethapyr to pea plants rather than its direct effect on rhizobia, since growth of rhizobia was not inhibited in both complex and defined media by concentrations of commercial or technical grade imazethapyr from 0.34 mM to 3.4 mM (Gonzalez *et al*., 1996). At sublethal doses of imazethapyr (0.7 mg L^{-1}), plants exhibited turgid root tips and short, thick roots with few abnormally curved lateral roots, and a lack of root hairs (Royuela *et al*., 2000). This alteration of root morphology may be detrimental to attachment of rhizobia and impede subsequent infection and establishment of the symbiosis. Similarly, nodule formation of alfalfa was inhibited by chlorsulfuron and no infection threads were visible (Martensson and Nilsson, 1989). It was concluded that chlorsulfuron causes root hair deformations which inhibit the normal root hair infection process, i.e. anchoring of rhizobia to the root hair, root hair deformation and infection thread formation.

Similar to Gonzalez *et. al.* (1996), Royuela *et al.* (2000) found that imazethapyr reduced the number of nodules on pea plants. However Royuela *et al.* (2000) also found reductions in total nodule mass and noted that the nodules formed in the presence of imazethapyr were 4 times larger than those on control plants. The specific nitrogen fixation rate as measured by acetylene reduction activity at the end of the four week study was significantly reduced by imazethapyr. This was despite similar carbohydrate availability to control plants. The inhibition of nitrogen fixation was not due to

decreased nodule occupancy by bacteroids since bacterial protein levels were not significantly reduced. The decline in specific nitrogen fixation appeared to be related to structural alterations caused by herbicide in terms of nodule morphology and bacteroid ultrastructure. In addition the larger sized nodules may have increased resistance to oxygen diffusion (Royuela *et al*., 2000).

Pre-exposure of rhizobia prior to inoculation has been studied by several authors with varying results. Gonzalez *et al*. (1996) found that pea plants inoculated with rhizobia grown in 0.34 mM imazethapyr (700 times the recommended field application rate) for 3 days had the same growth rate as plants inoculated with rhizobia grown without imazethapyr. The nodulation ability of pre-treated rhizobia was not affected, and plants from both treatments had the same organic nitrogen content (Gonzalez *et al*., 1996). Eberbach and Douglas (1989) found that concentrations of chlorsulfuron from 0.2 to 2 mg L^{-1} caused severe reductions in nodule numbers of subterranean clover. However inoculum grown in the presence of chlorsulfuron and repeatedly washed to remove associated carryover herbicide prior to inoculation of *Trifolium subterraneum* L. had no significant effect on plant nodulation.

In contrast to Gonazalez *et al*. (1996) and Eberbach and Douglas(1989), pre-exposure of rhizobia to chlorsulfuron or imazethapyr at recommended application rates reduced the number of nodules formed on chickpea by 51% and 35% respectively, in the absence of herbicide elsewhere in the system (Anderson, 2001). This indicated an effect on the ability of the rhizobia to form a symbiosis with the legume, despite no effect of the herbicide on growth of the rhizobia.

In summary there is evidence in the literature that nodule number is affected by AHASIH. There is consensus that the effects are on nodule initiation rather than development and it is usually attributed an affect of AHASIH on the plant. This is generally based on the lack of inhibition of growth and survival of rhizobia in the presence of the herbicides. While the growth and survival of chickpea rhizobia was not inhibited by AHASIH, nodulation of chickpea by pre-exposed rhizobia was (Anderson, 2001). This demonstrated that the ability of rhizobia to form a symbiosis was affected by AHASIH; a result that disputes the consensus and needs to be confirmed with a broader range of legumes and rhizobia.

Signalling

A complex series of interactions involving flavonoids from legumes and Nod metabolites from rhizobia are required for successful nodulation. There has been no work on the effect AHASIH may have on these interactions.

2.4 Summary and critical knowledge gaps

Group B herbicides have gained widespread use in Australian crops and pastures. Potential interactions of group B herbicides with legumes are by recommended in-crop applications directly to legume crops and pastures, as well as the persistence of the herbicides (the sulfonylurea class in particular) following application to previous cereal crops. Grain and pasture legumes are valued in farming systems for their ability to fix nitrogen in symbiosis with rhizobia. The inhibition of nitrogen fixation due to group B herbicides has been demonstrated for a limited range of grain and pasture legumes. Experimental work is required to assess whether group B herbicides impact on the nitrogen fixation of a broader range of grain and pasture legumes.

Most studies conclude that any inhibition of nitrogen fixation is due to herbicide impacts on the plant, rather than the rhizobia. This is because growth effects of the herbicides on rhizobia are only observed at very high levels of herbicide. However it has been demonstrated that exposure of rhizobia to herbicide prior to inoculation can have deleterious effects on nodulation and nitrogen fixation. Since the formation of effective symbioses is a complex process, there are many steps that might be compromised due to herbicide application. Abnormal root hairs have been observed on plants exposed to herbicide, with implications for root hair curling and infection by rhizobia. Accordingly, nodule initiation (manifested in a reduction in nodule number) has been identified as the reason for decreased nodulation due to group B herbicides. More work is required to improve our understanding of the mechanisms by which group B herbicides inhibit nitrogen fixation.

2.5 Thesis objectives

The main objectives of the work presented in this thesis were to:

1) Assess the impacts of recommended group B herbicides on the nitrogen fixation of pasture and grain legumes grown in southern Australia.

2) Determine the mechanisms of any reductions in nitrogen fixation resulting from application of group B herbicides.

Chapter 3 The impact of 'in-crop' group B herbicides on the growth, nodulation and nitrogen fixation of grain legumes

CHAPTER 3 THE IMPACT OF 'IN-CROP' GROUP B HERBICIDES ON THE GROWTH, NODULATION AND NITROGEN FIXATION OF GRAIN LEGUMES 68

3.1 Introduction

Herbicide recommendations and doses for use within legume crops and pastures are based on field trials that are primarily designed to determine whether yield penalties occur. Consequently herbicide labels often recognise symptoms such as leaf yellowing and stunting of growth, but the herbicide is generally considered safe if yield penalties are not measured. Thus herbicide recommendations do not consider potential impacts

on the amount of nitrogen fixed. Nitrogen fixation is an important agronomic attribute of legume crops and pastures and a major reason for the inclusion of legumes in farming systems. Therefore it is important to understand the effects that herbicides have on the legume / rhizobia symbiosis.

Given that evidence for the inhibition of nitrogen fixation by group B herbicides has been limited to experiments examining only a few legume / herbicide combinations, screening experiments were conducted to assess the scope of this problem. Grain legumes commonly grown in southern Australia and the group B herbicides recommended for 'in crop' use were screened for potential effects on nodulation and nitrogen fixation. A simple experimental system was used to identify reductions in nitrogen fixation in a herbicide by legume combination, and also provide some information about the mechanisms involved.

Previous work by Anderson *et al*. (2004) had shown reductions in the amount of nitrogen fixed by chickpea, associated not only with applications of group B herbicides to the plant, but also where the free-living rhizobia had been exposed to herbicide prior to inoculating the plants. It was found that chlorsulfuron, a sulfonylurea herbicide that is known to persist in soil following application to cereal crops, reduced the nodulation and nitrogen fixation of chickpea (Anderson *et al*., 2004). Growth of rhizobia *in vitro* with chlorsulfuron in the broth culture at rates thought to simulate field concentrations of herbicide reduced nodulation of chickpea by 51% compared to zero herbicide controls (Anderson *et al*., 2004). The screening work presented here incorporated a similar treatment in order to ascertain whether herbicide impacts on rhizobia contribute to reduced nitrogen fixation in other grain legumes.

The aim of the experiments described in this chapter was to assess recommended 'in crop' group B herbicides (flumetsulam, imazamox and imazethapyr) for impacts on the growth, nodulation and nitrogen fixation of field pea, chickpea, faba bean and vetch.

3.2 Materials and Methods

Experiments were set up in a factorial design, with three inoculation treatments and two plant applied herbicide treatments as follows:

3 inoculation treatments -

- Uninoculated (O)
- Washed rhizobia (-)
- Rhizobia grown in the presence of herbicide and washed (+)

2 herbicide treatments -

- Herbicide applied at the rate, method and stage recommended on the label (+)
- Zero herbicide control (-)

The rate of herbicide chosen was the maximum rate recommended on the herbicide label for the particular legume.

There were six replicates, with one plant per pot. Treatments were randomised within six blocks. To prevent edge effects, each line of pots (a block) had additional buffer plants on the ends of each row so that all plants within a block were surrounded by neighbouring pots. This ensured consistent shading and evaporation for all experimental pots within each block.

3.2.1 Inoculum

Certified commercial inoculum strains of rhizobia (see Table 3.2) were obtained from the Australian Legume Inoculants Research Unit (ALIRU) on agar slants. These were streaked onto yeast extract mannitol (YEM) plates containing 1% agar. Single colonies were selected and grown up in YEM broths (K_2HPO_4 2.87 mM, $MgSO_4.7H_2O$ 0.81 mM, NaCl 1.71 mM, mannitol 10 g L^{-1}, yeast extract 0.4 g L^{-1}) to make glycerol stocks. All plates and broth cultures were incubated at 25°C.

To characterise the growth of each strain, rhizobia were grown in 200 ml Erlenmeyer flasks containing 50 ml YEM broth. The absorbance of cultures at 500 *n*m (A500) was measured using a Beckman DU640 spectrophotometer every 4 hours. 1 in 10 dilution series of culture were made in phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4.2H_2O$ 4.3 mM, KH_2PO_4 1.4 mM) and drop plate counts (Miles and Misra, 1938) on YEM agar plates were used to calculate the number of colony forming

units (cfu). The growth curve for each *Rhizobium* strain was obtained, and the relationship between absorbance at 500 *n*m and the number of cfu derived.

To prepare inoculum for the screening experiments, flasks containing rhizobia defined media (Brown and Dilworth, 1975) were prepared in triplicate, with or without herbicide in the media. The rhizobia defined media consisted of KH_2PO_4 (2.65 mM), K_2HPO_4 (8.04 mM), $MgSO_4.7H_2O$ (1.01 mM), $CaCl_2.2H_2O$ (136.04 μM), NaCl (3.42 mM), KNO_3 (6.92 mM), $FeCl_3$ (40.69 μM), EDTA (40.3 μM), $ZnSO_4.7H_2O$ (556 nM), $NaMoO_4$ (1.09 μM), H_3BO_3 (4.04 μM), $MnSO_4.H_2O$ (896 nM), $CuSO_4.5H_2O$ (80.1 nM), $CoSO_4.6H_2O$ (3.8 nM) and filter sterilised thiamine HCl (2.96 μM), calcium pantothenate 4.2 μM), biotin (4.1 nM) and glucose (694 mM).

The amount of herbicide added to flasks was calculated on a surface area and volume basis (Table 3.1). For example, herbicide applied at the recommended rate per hectare and mixed evenly to a depth of 10 cm would exist in 10^6 litres of soil. An equivalent amount per cm^3 of soil was used per ml of culture. This calculation is conservative in that it does not account for the solid and air in the volume of soil. Therefore concentrations in the broth cultures are lower than potential concentrations in soil solution.
Twenty-four to 48 hours after seeding all broth cultures from a single starter culture, the optical densities at 500 *n*m were measured. Dilution series were made in PBS to determine the number of cfu in the culture by drop plate counts.

To ensure an equivalent number of rhizobia for each inoculation treatment, the number of cfu in each culture was estimated from the previously determined relationships between optical density of cultures at 500 *n*m and the cfu per ml of culture. A washing procedure was used to remove any residual herbicide from the inoculum. Cultures were centrifuged in sterile 30 ml centrifuge tubes by spinning at 10000 rpm for 10 minutes in a SS34 rotor and a Sorvall RC5C Plus or RC28S centrifuge. The supernatant was removed and the cells resuspended in PBS. The cells were centrifuged and resuspended two more times, with the last resuspension in a volume of PBS calculated to give an equivalent number of colony forming units for each rhizobia treatment, based on the estimate of the cfu per ml of culture described above. Pots were inoculated with approximately 10^3 cfu per gram of soil as described below.

Table 3.1 Recommended application rates of herbicide as the commercial formulation (imazethapyr 70% w/w as Spinnaker herbicide, flumetsulam 80% w/w as Broadstrike herbicide and imazamox 70% w/w as Raptor herbicide) and the amounts of commercial formulation used in the rhizobia cultures

Herbicide	Label rate for commercial formulation (g ha^{-1})	Amount per litre (µg)	Amount of commercial formulation per 50 ml culture (µg)	Amount of active ingredient per 50ml culture (µg)
Imazethapyr	45	45	2.25	1.58
Imazethapyr	70	70	3.5	2.45
Imazethapyr	100	100	5	3.5
Flumetsulam	25	25	1.25	1
Imazamox	45	45	2.25	1.25
Imazamox	50	50	2.5	1.75

3.2.2 Plant growth

Seeds within a 10% range by weight were used for any single experiment to minimise variability. Seed was sterilized for 1 minute in 70% ethanol, followed by 5 minutes in a 3% sodium hypochlorite solution. Seeds were then rinsed 8 times in sterile water, and 3 or more seeds transferred immediately to 1 litre WaterWell pots (Décor) filled with 1.5 kg of dried and sieved (to 4 mm) Golden Grove sand. The pots containing sand had been autoclaved previously and stored aseptically.

Rhizobial inoculum was applied by adding resuspended cells (described above) in sterile, quarter strength Tang's nutrient solution (Tang and Thomson, 1996) without nitrogen. 200 ml of nutrient solution containing the appropriate inoculation treatments was applied over the surface of the sand in each pot using squeeze bottles to give a uniform distribution. Tangs nutrient solution (KH_2PO_4 0.08 mM, K_2SO_4 2.4 mM, $MgSO_4.7H_2O$ 0.8 mM, $CaCl_2.2H_2O$ 2.4 mM, FeEDDHA 0.04 mM, H_3BO_3 0.02 mM, $Na_2MoO_4.2H_2O$ 0.12 µM, $ZnSO_4.7H_2O$ 3 µM, $MnSO_4.H_2O$ 4 µM, $CuSO_4.5H_2O$ 0.8 µM, $CoSO_4.7H_2O$ 0.8 µM) containing only a trace amount of nitrogen in the iron chelate was

chosen because it was suitable for adjusting the pH, contained chelated iron and was fully in solution.

Where herbicide was applied post-sowing pre-emergence (PSPE), it too was added to the nutrient solution. The amount of herbicide added for PSPE herbicide treatments was calculated on a pot surface area basis. After seeding and inoculation, machine clean recycled plastic beads were aseptically applied to the surface of pots. The beads minimised cross-contamination by rhizobia resulting from splashing during watering and reduced evaporation from the pots, thereby reducing the amount of watering required and the potential for contamination. Milli-Q water was used to bring pots to 60% water filled pore space. This was close to field capacity. Watering was done at least twice per week and more often in times of high evapotranspiration.

Pots were randomised within blocks and subjected to natural lighting in the glasshouse, with some attenuation using shade cloth for experiments conducted over the summer months. Temperatures were maintained at 25°C during the day and 15°C at night. After the seedlings had emerged they were thinned to one plant per pot, aiming for similarly sized seedlings within each block. Where the plant required physical support (e.g. peas and vetch), lengths of galvanised wire were used to form a frame.

The agricultural areas in which crop symptoms of group B herbicides have been noticed tend to have high pH soils. Therefore high pH was maintained by adjusting the pH of the nutrient solution and any water used to 8 with KOH.

3.2.3 Herbicide treatments

The legumes, herbicide rates and growth stage of herbicide application for each experiment are described in Table 3.2. Herbicide (Spinnaker containing 70% w/w imazethapyr, Raptor containing 70% w/w imazamox and Broadstrike containing 80% w/w flumetsulam) was applied with surfactants where recommended.

For post-emergent applications, herbicide was applied to plants using the Applied Molecular Ecology herbicide spray facility at the Waite campus, Adelaide, equipped with a calibrated boom and agricultural nozzles to deliver herbicide at the same droplet

size and concentration as would occur in the field. Since imazethapyr can be applied to peas as a post-sowing pre-emergence application or after emergence at the 4-6 node stage, both application times were tested in the same experiment.

Table 3.2 Inoculum strains and application rates for the herbicides as commercial formulations (imazethapyr 70% w/w as Spinnaker herbicide, flumetsulam 80% w/w as Broadstrike herbicide and imazamox 70% w/w as Raptor herbicide) and active ingredient in parentheses. BS1000 is a non-ionic surfactant. The plant growth stage for herbicide application and harvest (days after application) are also given. PSPE refers to a post-sowing, pre-emergence application.

Legume	Inoculum	Herbicide	Rate (g/ha)	Additive	Growth Stage	Timing/ Harvest
Chickpea *Cicer arietinum* Howzat	N CC1192	Imazethapyr	45 (31.5 ai)		PSPE	4 weeks duration
		Flumetsulam	25 (20 ai)		4-6 branch	17 days after application
Pea *Pisum sativum* Parafield	E SU303	Imazamox	45 (31.5 ai)	BS1000	to 4 node	11 days after application
		Flumetsulam	25 (20 ai)		2-6 node	13 days after application
		Imazethapyr	100 (70 ai)		PSPE	5 weeks duration
		Imazethapyr	70 (49 ai)	BS1000		17 days after application
Faba bean *Vicia faba* Fiesta	F WSM1455	Imazethapyr	100 (70 ai)		PSPE	6 weeks duration
Vetch *Vicia sativa* Morava	E SU303	Flumetsulam	25 (20 ai)		3 leaf to flowering	9 days after application

3.2.4 Harvest

Experiments were harvested at least 10 days after post emergent herbicide applications. For post sowing pre-emergent applications, 4 to 6 weeks of plant growth was allowed before harvest. Plants were carefully removed from pots and sand washed off roots using a gentle stream of water over a 2mm sieve so as to minimise the loss of any root material or nodules. Shoots where immediately separated from roots by cutting with a scalpel blade at a uniform position on each plant, and samples were placed into bags for drying or nodule assessment.

To facilitate manipulation of roots for the removal of nodules, samples were placed in a large glass petrie dish containing water. Black or white paper was used as a background under the petrie dishes to provide maximal contrast with the spectrum of shades and colours of the nodules. Nodules were removed using forceps and in most cases were placed into 'functional' and 'non-viable' piles based on their appearance (pink and non-pink respectively). Nodules were then counted into small paper envelopes for each plant. Where pink and non-pink nodules were separated these were counted and weighed individually, or else pooled where only total nodulation was assessed. Shoot, root and nodule samples were dried at 55-60°C to constant weight (at least 48 hours). Dry shoot, root and nodule samples were weighed.

3.2.5 Nitrogen analysis

Nitrogen analysis was done using Kjeldahl digests. Nodules for each plant were placed in digest tubes with the roots. Shoots from each plant were placed into separate tubes. Digests were set up by adding high selenium Kjeldahl tablets (Labchem) to samples in Pyrex tubes, pre-scored at 50 ml. 4 ml of 98% technical grade sulfuric acid was added to each tube, and then two lots of 1 ml of 50% cosmetic grade peroxide added slowly as the addition of peroxide creates a vigorous reaction. A laboratory standard (3.6% organic nitrogen in ground grass clippings) was included to confirm the recovery of organic nitrogen from each digest run. Digests were carried out on heating blocks in hoods with NaOH scrubbers for 30 minutes at 200ºC and 180 minutes at 330ºC. $(NH_4)_2SO_4$ was made up in blank digest solution to give standards containing 0, 5, 10, 15 and 20 μg ml^{-1} of nitrogen. Digested material was diluted first to 50 ml in the Pyrex tubes, and then as required into the sample tubes for the auto analyser. Samples were

run on a segmented flow auto analyser along with the standards. The auto analyser combined the sample with aqueous solutions of sodium nitroprusside (0.2 g L^{-1}), sodium dichloroisocyanurate (0.1 g with 20 g NaOH to 1 litre), sodium salicylate (10 g L^{-1}) and citrate-tartrate complex (25 g NaOH, 6 g sodium citrate, 18 g sodium hydrogen tartrate, 0.25 ml of 30% BRIJ to 1 litre). Peaks were manually measured from a chart produced by an analogue chart recorder. A regression of the peak height for the standards against their nitrogen content was calculated. The amount of nitrogen for each sample was then calculated from the peak height, the standard regression coefficient and the dilution factor for each sample.

3.2.6 Calculations and statistical analysis

Most variates were directly measured (dry weight and nitrogen contents, nodule counts). Some relationships and ratios were obtained using simple calculations (e.g. number of nodules per unit root dry weight was used to account for the effect of variation in root biomass on nodulation). An index of the relative amount of root and shoot biomass and nitrogen was calculated by dividing the root plus nodule dry weight by the shoot dry weight.

The amount of nitrogen fixed by inoculated plants was calculated by subtracting the amount of nitrogen in the uninoculated plant from the amount of nitrogen in the inoculated plant with the same plant applied herbicide treatment within each block. This accounted for the seed nitrogen, or any trace amounts of nitrogen in the growth media. It was assumed that any amount of nitrogen in inoculated plants greater than that of uninoculated plants had come from nitrogen fixation, and that losses of nitrogen from plants (e.g. ammonia volatilisation and leaf drop) were equivalent in the inoculated and uninoculated plants.

Data were analysed by analysis of variance using two models:

ANOVA 1

Treatment structure: plant herbicide * rhizobia pre-treatment.

Data: Inoculated plants only. All variates.

The ANOVA 1 treatment structure was used for analysing all variates, alongside the nested ANOVA described below, because it gave analyses that included only inoculated plants and was therefore more relevant to field grown crops. Also, the rhizobia treatment in the ANOVA 1 structure was purely a herbicide pre-exposure one, and the restricted ANOVA more clearly identified significant effects and interactions due to exposure of rhizobia to herbicide.

A nested ANOVA (ANOVA 2) was performed on all plant growth data. In this analysis the rhizobia herbicide treatment was nested within the inoculation treatment:

ANOVA 2

Treatment structure: plant herbicide * inoculation / rhizobia pre-exposure
Data: All plants. Plant growth and nitrogen data only.

This nested ANOVA had the ability to detect main effects of plant applied herbicide including plant effects on uninoculated plants and therefore was able to identify herbicide treatment effects that occurred independent of symbiotic nitrogen fixation. The identification of plant growth effects in uninoculated plants was possible in the grain legume experiments due to the substantial seed nitrogen that allowed sufficient growth of the plants to detect repression by herbicide treatment. The nested ANOVA was only applied to plant growth data, not the nodulation and nitrogen fixation data since the inclusion of uninoculated plants in such an analysis was meaningless and violated the assumptions of ANOVA.

Plots of residuals were examined for homogeneity of variance and data transformed where required.

All statistical analyses were completed using Genstat 5 (Release 4.1 1997 VSN International Ltd: Hemel Hempstead, UK).

Data from individual experiments are presented in tables to be found at the end of each section. Significant differences ($p<0.05$) are presented, along with trends ($0.05<p<1$) where they convey useful information.

For ANOVA 1, only inoculated plants are included in the analysis. Where there was a main effect of plant-applied herbicide, the herbicide treatments are denoted by '**-**'or '**+**', and '**I**' is used to indicate all inoculated plants. Where the rhizobia pre-treatment effect was significant, the unexposed rhizobia are indicated by '**-**'and the pre-exposed rhizobia by '**+**'.

For ANOVA 2, the inoculation treatments are denoted by '**U**' for uninoculated, and '**I**' for inoculated or combined '**U and I**' where there was a main effect of plant-applied herbicide. Where the nested rhizobia pre-treatment needs to be denoted, **O** is used for uninoculated, the unexposed rhizobia are indicated by '**-**'and the pre-exposed rhizobia by '**+**'. The plant-applied herbicide treatments are '**-**' or '**+**', or combined where there was a main effect of rhizobia treatment.

3.3 Results

There were no significant effects of any of the herbicides on any of the rhizobia strains grown *in vitro*, as measured by optical density of cultures and drop plate counts of the number of colony forming units. It can therefore be assumed that group B herbicides did not affect the growth of rhizobia *in vitro* at the concentrations tested. These results are not presented.

A summary of the treatment effects on plant parameters are presented in Table 3.3. A variety of responses in the seven herbicide by legume combinations were observed. These ranged from no significant effects of herbicide on any of the parameters measured, to clear effects of the plant-applied herbicide on growth, nodulation and nitrogen fixation. These are described in more detail for each experiment.

Table 3.3 Treatment effects observed in the screening experiments. ANOVA 1 excludes uninoculated plants having treatment structure: herbicide*rhizobia pre treatment. ANOVA 2 (in brackets) includes uninoculated plants with treatment structure herbicide*inoculation / rhizobia pre-treatment. Significant effects are given ($p<0.05$ in bold, $0.05<p<0.1$ in grey). H=plant applied herbicide treatment; I=inoculation treatment; R=rhizobia pre-treatment; Blank cell indicates no significant treatment effects.

Summary of treatment effects on inoculated plants ANOVA 1 (ANOVA 2)	Chickpea Imazethapyr	Chickpea Flumetsulam	Pea Imazamox	Pea Imazethapyr	Pea Flumetsulam	Bean Imazethapyr	Vetch Flumetsulam
Total plant nitrogen (mg)	H (H*I)	H*R	H H*R (H*I/R)	(I)	(I)	(I)	H (I)
Total plant dry weight (g)	H (H*I)		H (I)		(I)	(I)	H (I)
Total plant nitrogen concentration (mg g^{-1})	(H*I)	H*R (I)	(H*I)	H (I)	(I) (H*I)	R R*H	H (I)
Shoot nitrogen (mg)	(H*I)	(I)	H H*R (H*I/R)	(I)	(I)	(I)	H (I)
Shoot dry weight (g)	(H*I)	H (I)	H (I)	(I)	(I)	(I)	H (I)
Shoot nitrogen concentration (mg g^{-1})	(I)	H (I)	(H*I)	H (I)	(I) (H)	(I)	H (I)
Root + Nodule nitrogen (mg)	H (H*I)	H (I)	H*R (H*I/R)	(I)	(I)	R (R)	(I)
Root + Nodule dry weight (g)	H (I)	H (I)	(I)	(I)	(I)	(I)	(I)
Root + Nodule nitrogen concentration (mg g^{-1})	(I)	(I)	(I)	(I)	(H*I)	R R*H	(I)
Root : Shoot dry weight ratio	H (H*I)	H (I)	H (I)	(I)	(I)	R (I)	H (I)
Root : Shoot nitrogen ratio	H (I)	H (I)	H R	(I)	(I)	R (I)	H H*R (I)
Nodule dry weight (g)	H						
Nodule number	H			H			H
Dry weight per nodule (g $nodule^{-1}$)	H		H		H		
Nodule dry weight per unit root dry weight (g g^{-1})	H						H
Nodule number per unit root dry weight (g^{-1})				H			H
Amount of nitrogen fixed (mg)	H		H		H		H
Nitrogen fixed per unit nodule dry weight (mg g^{-1})	H		H		H		H
Nitrogen fixed per nodule (mg $nodule^{-1}$)	H	R	H				H

The major findings from each experiment are described below. Refer to the tables in each section for treatment effects and means, as well as standard deviations are presented for main effects and least significant differences where more than two treatment effects need to be compared.

3.3.1 Chickpea / Imazethapyr (Table 3.4)

Imazethapyr was applied to chickpea plants as a post-sowing pre-emergence (PSPE) application. The herbicide was therefore present in the potting mix for the entire experiment excluding the initial imbibing that occurred during sterilization of the seed. Also, more herbicide was expected to be in direct contact with and taken up through the roots in comparison to shoot applied herbicide applications. Accordingly large reductions in the dry weight of below ground organs were observed in this experiment. For inoculated plants, the decrease in the dry weight of roots plus nodules of 41% with herbicide compared with no herbicide was matched by an equivalent reduction in below ground nitrogen.

PSPE application of imazethapyr did not affect the shoot dry weight of inoculated chickpea plants, but increased the shoot dry weight of uninoculated plants. It is conceivable that with the large reductions in root biomass, more resources were available for shoot growth. This result may also suggest that translocation of herbicide from roots to shoot meristems was limited.

Uninoculated plants had a higher proportion of biomass in roots than inoculated plants. This may be symptomatic of the need to obtain additional sources of nitrogen, either from the growth medium, or by establishing symbioses with soil rhizobia. Consistent with the large reductions in root growth, imazethapyr reduced the proportion of biomass in below ground organs, but to a greater extent for uninoculated plants than for inoculated plants.

Imazethapyr did not affect the nitrogen concentrations of shoots or below ground organs, whereas inoculation with rhizobia increased nitrogen concentrations of roots and shoots. However when above and below ground data were pooled to give total plant data, an interaction between inoculation and plant applied herbicide was evident.

For uninoculated plants an increase in nitrogen concentration with herbicide application was observed. Total plant nitrogen in uninoculated plants theoretically came solely from the seed nitrogen reserve. Accordingly the total amount of nitrogen in uninoculated plants did not change with herbicide application. Therefore the reduction in plant dry weight due to herbicide corresponded with an increase in nitrogen concentration. In contrast, inoculated plants had a source of nitrogen from symbiotic nitrogen fixation. For these plants, the reduction in dry weight due to plant applied herbicide was matched by a decrease in plant nitrogen so the nitrogen concentration of roots or shoots did not change. This result also suggests that nitrogen fixation was not affected independent of plant growth because a reduction in nitrogen concentration was not observed.

Total nodule number, total nodule dry weight and dry weight per nodule were reduced by plant applied herbicide. Therefore not only were there fewer nodules, they were also smaller. When expressed on a root dry weight basis, a significant decrease in nodule dry weight per unit root dry weight due to herbicide application was found, but there was no significant reduction in the number of nodules per unit root dry weight. Therefore it appears that nodule development, rather than initiation was reduced. A visual assessment of nodules revealed that for those plants treated with herbicide, nodules were less pink in colour and had a different surface morphology compared to controls.

Plant applied herbicide reduced the calculated amount of nitrogen fixed by 60%. Imazethapyr also reduced the amount of nitrogen fixed per unit nodule dry weight by 28%, and per nodule by 44%.

There were no rhizobia pre-exposure effects measured in this experiment. Furthermore, imazethapyr reduced the dry weight of uninoculated chickpea plants indicating a definite plant growth effect. The fact that the number of nodules per unit root dry weight did not decrease with plant applied herbicide indicates that the reduction in nodule number was directly linked to reductions in root growth. Reduced nodulation was caused by the inhibition of nodule development as evidenced by reduced dry weight per nodule and also reduced nodule dry weight per unit root dry weight.

Table 3.4 Treatment effects and interactions for the chickpea / imazethapyr experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Chickpea (Howzat) / Imazethapyr post-sowing pre-emergence							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
p=<0.001	I	-	12.45 (1.03)	p<0.001	U	-	7.61 (0.06) **a**
	I	+	9.50 (1.16)	lsd 1.098	U	+	7.57 (0.09) **a**
					I	-	12.45 (1.03) **b**
					I	+	9.50 (1.16) **c**
Total plant dry weight (g)							
p=<0.001	I	-	0.5084 (0.0321)	p=0.041	U	-	0.4664 (0.0201) **a**
	I	+	0.3751 (0.0424)	lsd 0.0336	U	+	0.3762 (0.0390) **b**
					I	-	0.5084 (0.0321) **c**
					I	+	0.3751 (0.0424) **b**
Total plant nitrogen concentration (mg g^{-1})							
ns				p=0.025	U	-	16.33 (1.45) **a**
				lsd 2.141	U	+	20.17 (1.66) **b**
					I	-	24.56 (2.32) **c**
					I	+	25.36 (1.87) **c**
Shoot nitrogen (mg)							
ns				p=0.005	U	-	3.85 (0.69) **a**
				lsd 0.908	U	+	5.01 (0.67) **b**
					I	-	6.36 (0.08) **c**
					I	+	5.86 (0.10) **bc**
Shoot dry weight (g)							
ns				p=0.011	U	-	0.1649 (0.0148) **a**
				lsd 0.02265	U	+	0.1942 (0.0180) **b**
					I	-	0.2085 (0.0218) **b**
					I	+	0.2008 (0.0279) **b**
Shoot nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	24.58 (3.29)
					I	- and +	29.85 (2.96)
Root + Nodule nitrogen (mg)							
p<0.001	I	-	6.091 (0.496)	p<0.001	U	-	3.757 (0.415) **a**
	I	+	3.640 (0.537)	lsd 0.5382	U	+	2.554 (0.416) **b**
					I	-	6.091 (0.495) **c**
					I	+	3.640 (0.536) **a**
Root + Nodule dry weight (g)							
p<0.001	I	-	0.2999 (0.0285)	p<0.001	U and I	-	0.3005 (0.0254)
	I	+	0.1743 (0.0196)		U and I	+	0.1769 (0.0217)
Root + Nodule nitrogen concentration (mg g^{-1})							
				p<0.001	U	- and +	13.29 (1.69)
					I	- and +	20.69 (2.31)

Chickpea (Howzat) / Imazethapyr post-sowing pre-emergence							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Root : Shoot dry weight ratio							
p<0.001	I	-	1.345 (0.234)	p=0.005	U	-	1.843 (0.225) **a**
	I	+	0.820 (0.122)	lsd 0.1792	U	+	0.938 (0.122) **b**
					I	-	1.345 (0.233) **c**
					I	+	0.812 (0.122) **b**
Root + Nodule : Shoot nitrogen ratio							
p<0.001	I	-	0.971 (0.147)	p<0.001	U and I	-	0.985 (0.184)
	I	+	0.644 (0.188)		U and I	+	0.601 (0.172)
Nodule dry weight (mg)							
p<0.001	I	-	23.17 (3.91)	na			
	I	+	11.82 (2.99)				
Nodule number							
p=0.065	I	-	10.29 (5.54)	na			
	I	+	7.21 (3.87)				
Dry weight per nodule (mg nodule^{-1})							
p=0.03	I	-	2.66 (0.04)	na			
	I	+	1.89 (0.02)				
Nodule dry weight per unit root dry weight (g g^{-1})							
p=0.05	I	-	0.0844 (0.0165)	na			
	I	+	0.0729 (0.0168)				
Nodule number per unit root dry weight (g^{-1})							
ns				na			
Amount of nitrogen fixed (mg)							
p<0.001	I	-	4.85 (1.02)	na			
	I	+	1.93 (1.18)				
Nitrogen fixed per unit nodule dry weight (mg g^{-1})							
p=0.05	I	-	218 (73)	na			
	I	+	158 (93)				
Nitrogen fixed per nodule (mg nodule^{-1})							
p=0.012	I	-	0.559 (0.250)	na			
	I	+	0.314 (0.248)				

3.3.2 Chickpea / Flumetsulam (Table 3.5)

In contrast to imazethapyr, flumetsulam was applied directly to chickpea plant shoots as a post emergence application. In this experiment there were no herbicide induced changes to total plant dry weight, however biomass partitioning was affected by plant applied flumetsulam with increased shoot dry weight and decreased root dry weight compared to zero herbicide plants. This was despite flumetsulam being applied onto the shoots at the 4 to 6 branch stage. Root plus nodule dry weight and nitrogen was

reduced by herbicide application across all inoculation treatments. Despite the impacts on root growth, there were no significant effects of herbicide on nodulation.

A herbicide by rhizobia interaction affected total plant N. Unsprayed plants inoculated with pre-exposed rhizobia had higher plant nitrogen than those inoculated with unexposed rhizobia. This result contradicted the hypothesis that herbicide impacts on rhizobia and hence reduces nitrogen fixation.

There were no significant effects of herbicide on the calculated amount of nitrogen fixed. However a trend was observed (p=0.083) in which plant applied herbicide increased the amount of nitrogen fixed per nodule.

Table 3.5 Treatment effects and interactions for the chickpea / flumetsulam experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Chickpea (Howzat) / Flumetsulam							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
p=0.022	-	-	12.89 **a**	p=0.052	O	-	8.47
lsd 1.407	-	+	13.92 **ab**	lsd 1.625	O	+	8.32
	+	-	14.79 **bc**		-	-	13
	+	+	13.43 **ab**		-	+	13.92
					+	-	14.79
					+	+	13.43
Total plant dry weight (g)			ns		ns		
Total plant nitrogen concentration (mg g^{-1})							
p=0.077	-	-	21.84	p<0.001	U	- and +	14.43 (3.21)
lsd 1.973	-	+	23.45		I	- and +	23.27 (1.93)
	+	-	24.18				
	+	+	23.3				
Shoot nitrogen (mg)							
ns				p<0.001	U	- and +	4.57 (1.25)
					I	- and +	8.11 (1.10)
Shoot dry weight (g)							
p=0.012	I	-	0.2612 (0.0208)	p=0.002	U and I	-	0.2421 (0.0338)
	I	+	0.296 (0.0368)		U and I	+	0.2776 (0.0470)

Chickpea (Howzat) / Flumetsulam							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Shoot nitrogen concentration (mg g^{-1})							
p=0.052	I	-	30.11 (3.43)	p<0.001	U	- and +	20.53 (4.05)
	I	+	28.25 (1.71)		I	- and +	29.18 (2.83)
Root + Nodule nitrogen (mg)							
p=0.069	-	-	5.89	p<0.001	U and I	-	5.54 (1.22)
lsd 0.806	-	+	5.53		U and I	+	4.68 (1.16)
	+	-	6.52	p<0.001	U	- and +	3.83(0.79)
	+	+	5.12		I	- and +	5.77 (0.89)
p=0.005	I	-	6.22 (0.77)				
	I	+	5.33 (0.79)				
Root + Nodule dry weight (g)							
p=0.001	I	-	0.3429 (0.0464)	p<0.001	U and I	-	0.3520 (0.052)
	I	+	0.2894 (0.0339)		U and I	+	0.3142 (0.051)
				p=0.004	U	- and +	0.3671 (0.051)
					I	- and +	0.3162 (0.048)
Root + Nodule nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	10.7 (2.95)
					I	- and +	18.36 (2.34)
Root : Shoot dry weight ratio							
p<0.001	I	-	1.221 (0.160)	p<0.001	U and I	-	1.418 (0.365)
	I	+	0.911 (0.151)		U and I	+	1.123 (0.367)
				p<0.001	U	- and +	1.680 (0.330)
					I	- and +	1.066 (0.219)
Root : Shoot nitrogen ratio							
p<0.001	I	-	0.811 (0.085)	p<0.001	U and I	-	0.890 (0.18)
	I	+	0.645 (0.115)		U and I	+	0.668 (0.13)
				p=0.005	U	- and +	0.880 (0.25)
					I	- and +	0.729 (0.13)
Nodule dry weight (g) ns					na		
Nodule number ns					na		
Dry weight per nodule (g nodule^{-1}) ns					na		
Nodule dry weight per unit root dry weight (g g^{-1}) ns					na		
Nodule number per unit root dry weight (g^{-1}) ns					na		
Amount of nitrogen fixed (mg) ns					na		
Nitrogen fixed per unit nodule dry weight (mg g^{-1}) ns					na		
Nitrogen fixed per nodule (mg nodule^{-1})					na		
p=0.083	-	- and +	0.42 (0.21)				
	+	- and +	0.556 0.22)				

3.3.3 Pea / Imazamox (Table 3.6)

Post emergence application of imazamox to pea plants at the 4 node stage reduced the total dry weight of pea plants by reducing shoot growth by 24%. Root plus nodule dry weight was unaffected by plant applied herbicide. Accordingly, there was a greater allocation of biomass to below ground organs.

An interaction between plant-applied herbicide and the inoculation treatments was found to affect total plant nitrogen. The herbicide treatment did not affect the amount of nitrogen in uninoculated plants. All inoculated plants accumulated more nitrogen than the uninoculated plants, indicating that nitrogen fixation had occurred. For plants inoculated with unexposed rhizobia, plant applied herbicide reduced the amount of nitrogen from 13.3 to 10.0 mg per plant. The amount of nitrogen in plants inoculated with pre-exposed rhizobia was intermediate to these values. Herbicide application to these plants did not significantly change the amount of nitrogen per plant with unsprayed plants having 12.2 mg and sprayed plants 11.4 mg per plant. The nitrogen contents of shoots and below ground samples followed similar trends. That is, there were significant declines in the amount of nitrogen in shoots and below ground samples when herbicide was applied to plants inoculated with unexposed rhizobia; however herbicide application had no effect on the amount of nitrogen in shoots and below ground samples of plants inoculated with pre-exposed rhizobia.

An increase in the ratio of root to shoot dry weight due to plant applied herbicide reflected the decrease in shoot dry weight given the absence of a significant change in root dry weight compared to control plants. Uninoculated plants had a much larger proportion of dry weight in roots compared with inoculated plants. However there was a different scenario for the partitioning of nitrogen. While herbicide application increased the relative amount of nitrogen in the below ground sample, pre-exposure of rhizobia to herbicide was found to reduce the proportion of nitrogen in the below ground sample.

Despite the treatment effects on plant nitrogen status, there were no significant changes to total nodule number or dry weight. However, plant-applied herbicide appeared to reduce the dry weight per nodule by 24% ($p=0.06$).

Imazamox application reduced the amount of nitrogen fixed by 45%. Given that there were no significant differences in nodulation, it follows that the amount of N_2 fixed per unit nodule dry weight declined by 47% and the amount of N_2 fixed per nodule declined by 59% due to herbicide application. Thus it was demonstrated that application of a group B herbicide at the four node stage, at which time substantial nodulation may have already occurred, can still reduce the amount of nitrogen fixed.

Table 3.6 Treatment effects and interactions for the pea / imazamox experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Pea (Parafield) / Imazamox							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
p=0.007	I	-	12.73 (1.68)	p=0.008	O	-	7.2 **a**
	I	+	10.72 (1.87)	lsd 1.639	O	+	7.65 **a**
p=0.076	-	-	13.25		-	-	13.28 **c**
lsd 1.972	-	+	9.99		-	+	9.99 **b**
	+	-	12.20		+	-	12.20 **bc**
	+	+	11.44		+	+	11.44 **b**
Total plant dry weight (g)							
p=0.009	I	-	0.528 (0.080)	p=0.006	U and I	-	0.503 (0.081)
	I	+	0.449 (0.061)		U and I	+	0.438 (0.069)
				p=0.024	U	- and +	0.433 (0.072)
					I	- and +	0.489 (0.080)
Total plant nitrogen concentration (mg g^{-1})							
ns				p=0.047	U	-	15.94 **a**
					U	+	18.89 **b**
					I	-	24.28 **c**
					I	+	23.86 **c**
				p=0.092	O	-	15.94
				lsd 2.713	O	+	18.89
					-	-	24.97
					-	+	23.67
					+	-	23.59
					+	+	24.06
Shoot nitrogen (mg)							
p<0.001	I	-	6.38 (1.05)	p=0.004	O	-	2.68 **a**
	I	+	4.63 (1.13)	lsd 1.624	O	+	2.68 **a**
p=0.09	-	-	6.55		-	-	6.55 **c**

Pea (Parafield) / Imazamox							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
lsd 1.196	-	+	4.04		-	+	4.04 **ab**
	+	-	6.22		+	-	6.22 **c**
	+	+	5.19		+	+	5.19 **bc**
Shoot dry weight (g)							
p=0.002	I	-	0.2682 (0.0541)	p<0.001	U and I	-	0.2339 (0.0697)
	I	+	0.2038 (0.0398)		U and I	+	0.1836 (0.0484)
				p<0.001	U	- and +	0.1533 (0.0393)
					I	- and +	0.2364 (0.0561)
Shoot nitrogen concentration (mg g^{-1})							
ns				p=0.017	U	-	16.72 **a**
					U	+	19.22 **b**
					I	-	24.02 **c**
					I	+	22.63 **c**
Root + Nodule nitrogen (mg)							
p=0.082	-	-	6.72	p=0.048	O	-	4.52 **a**
lsd 0.852	-	+	5.92	lsd 0.755	O	+	4.97 **a**
	+	-	5.99		-	-	6.72 **c**
	+	+	6.25		-	+	5.92 **b**
					+	-	5.99 **bc**
					+	+	6.25 **bc**
Root + Nodule dry weight (g)							
ns				p=0.018	U	- and +	0.2797 (0.038)
					I	- and +	0.2526 (0.029)
Root + Nodule nitrogen concentration (mg g^{-1})							
ns					U	- and +	17.22 (3.38)
					I	- and +	24.75 (2.66)
Root : Shoot dry weight ratio							
p<0.001	I	-	9.67 (0.144)	0.005	U and I	-	1.245 (0.476)
	I	+	1.203 (0.166)		U and I	+	1.460 (0.440)
				p<0.001	U	- and +	1.899 (0.353)
					I	- and +	1.079 (0.257)
Root : Shoot nitrogen ratio							
p<0.001	I	-	1.008 (0.133)	p<0.001	U and I	-	1.230 (0.350)
	I	+	1.357 (0.227)		U and I	+	1.542 (0.386)
p=0.005	-	- and +	1.271 (0.306)	p<0.001	O	- and +	1.8
	+	- and +	1.093 (0.159)	lsd 0.1699	-	- and +	1.265
					+	- and +	1.093
Nodule dry weight (g)			ns	na			
Nodule number			ns	na			
Dry weight per nodule (mg $nodule^{-1}$)				na			
p=0.06	I	-	0.123 (0.051)				
	I	+	0.094 (0.032)				
Nodule dry weight per unit root dry weight (g g^{-1})							
			ns	na			

Pea (Parafield) / Imazamox							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nodule number per unit root dry weight (g^{-1})							
			ns	na			
Amount of nitrogen fixed (mg)				na			
p=0.003	I	-	5.54 (1.92)				
	I	+	3.07 (2.09)				
p=0.091	-	-	6.08				
lsd 2.096	-	+	2.34				
	+	-	5.01				
	+	+	3.79				
Nitrogen fixed per unit nodule dry weight (mg g^{-1})							
p<0.01	I	-	1062 (372)	na			
	I	+	567 (286)				
Nitrogen fixed per nodule (mg $nodule^{-1}$)				na			
p<0.01	I	-	0.1317 (0.0688)				
	I	+	0.0534 (0.0290)				

3.3.4 Pea / Flumetsulam (Table 3.7)

Visual observations of the plants in this experiment did reveal some yellowing of leaves at the time of harvesting. Accordingly, there was a trend (p=0.058) for reduced shoot nitrogen concentrations due to herbicide application. Herbicide application increased the below ground nitrogen concentrations of uninoculated plants, but not inoculated plants. There was also little impact of herbicide on nodulation. There was a trend for increased dry weight per nodule (p=0.058) where plants were inoculated with rhizobia that were pre-exposed to the herbicide.

Despite the lack of large measurable effects of flumetsulam on the growth and nodulation of pea plants, there was a 31% reduction in the amount of N_2 fixed, and a 41% reduction in the amount of N_2 fixed per unit nodule dry weight. Thus even with only a few subtle visual symptoms, flumetsulam still impacted on the amount of nitrogen fixed by pea plants.

Table 3.7 Treatment effects and interactions for the pea / flumetsulam experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Pea (Parafield) / Flumetsulam							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
ns				p<0.001	U	- and +	7.39 (1.17)
					I	- and +	11.97 (2.15)
Total plant dry weight (g)							
ns				p=0.019	U	- and +	0.468 (0.071)
					I	- and +	0.546 (0.120)
Total plant nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	15.88 (1.80)
					I	- and +	22.20 (2.37)
				p=0.079	U	-	15.27
					U	+	16.48
					I	-	22.62
					I	+	21.79
Shoot nitrogen (mg)							
ns				p<0.001	U	- and +	2.7 (0.79)
					I	- and +	5.8 (1.81)
Shoot dry weight (g)							
ns				p<0.001	U	- and +	0.18 (0.028)
					I	- and +	0.298 (0.105)
Shoot nitrogen concentration (mg g^{-1})							
ns				p=0.058	U and I	-	18.98 (3.74)
					U and I	+	17.61 (3.97)
				p<0.001	U	- and +	15.07 (3.72)
					I	- and +	19.9 (2.87)
Root + Nodule nitrogen (mg)							
ns				p<0.001	U	- and +	4.69 (0.81)
					I	- and +	6.17 (0.96)
Root + Nodule dry weight (g)							
				p=0.004	U	- and +	0.288 (0.046)
					I	- and +	0.2472 (0.0329)
Root + Nodule nitrogen concentration (mg g^{-1})							
ns				p=0.048	U	-	14.92 a
					U	+	17.89 b
					I	-	24.93 c
					I	+	24.99 c
Root : Shoot dry weight ratio							
ns				p<0.001	U	- and +	1.612 (0.195)
					I	- and +	0.889 (0.279)

plant herbicide * rhizobia pre-treatment				plant herbicide*inoculation/rhizobia pre-treatment			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Root : Shoot nitrogen ratio							
ns				p<0.001	U	- and +	1.837 (0.461)
					I	- and +	1.141 (0.312)
Nodule dry weight (g)			ns	na			
Nodule number			ns	na			
Dry weight per nodule (mg nodule^{-1})							
p=0.058	-	- and +	0.198 (0.185)				
	+	- and +	0.308 (0.255)				
Nodule dry weight per unit root dry weight (g g^{-1})			ns	na			
Nodule number per unit root dry weight (g^{-1}) ns				na			
Amount of nitrogen fixed (mg)				na			
p=0.016	I	-	5.47 (2.20)				
	I	+	3.77 (2.61)				
Nitrogen fixed per nodule (mg nodule^{-1}) ns				na			
Nitrogen fixed per unit nodule dry weight (mg g^{-1})							
p=0.005	I	-	955 (353)	na			
	I	+	560 (402)				

3.3.5 Pea / Imazethapyr (Table 3.8)

In accordance with herbicide labels, imazethapyr was applied to pea plants post-sowing pre-emergence (PSPE), and also post emergence within the one experiment. In the statistical analysis there were three herbicide treatments (control, pre and post). As in the pea / flumetsulam experiment, there were few obvious impacts of imazethapyr on plant growth. However some trends were evident. The pre-emergence application of herbicide increased the total plant N concentration of inoculated and uninoculated plants to 19.5 mg g^{-1}, compared with 17.5 mg g^{-1} for unsprayed and post-emergent applications (p=0.051).

There was a significant reduction in nodule number in plants where imazethapyr was applied PSPE (12.4) compared with the non-herbicide control (18.4), or when herbicide was applied at the 4-6 node stage (24.2). Thus the post-emergent application increased the number of nodules per plant compared with zero herbicide controls. When expressed on a root dry weight basis a similar trend was found. That is, the PSPE herbicide application reduced the number of nodules per unit root dry weight compared

to no herbicide controls, and post-emergent application of imazethapyr increased the number of nodules per unit root dry weight.

Despite these changes to nodulation, there were no significant effects on the calculated amount of nitrogen fixed. Given the changes to nodulation observed, it may be expected that over time, differences in the amount of nitrogen fixed would become more apparent. However it is also possible that compensation can occur with changes in the efficiency of those nodules, or recovery might occur.

Table 3.8 Treatment effects and interactions for the pea / imazethapyr experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Pea (Parafield) / Imazethapyr							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
ns				p<0.001	U	- and +	5.51 (1.04)
					I	- and +	7.26 (1.82)
Total plant dry weight (g)				ns			
Total plant nitrogen concentration (mg g^{-1})							
0.087	I	-	18.46 (2.79)	0.051	U and I	-	17.27 (3.27)
	I	post	18.73 (3.34)		U and I	post	17.50 (3.49)
	I	pre	21.21 (3.65)		U and I	pre	19.50 (3.93)
				p<0.001	U	- and +	15.34 (2.32)
					I	- and +	19.47 (3.44)
Shoot nitrogen (mg)							
ns				p<0.001	U	- and +	2.94 (0.81)
					I	- and +	4.33 (1.38)
Shoot dry weight (g)							
ns				p<0.001	U	- and +	0.1732 (0.0271)
					I	- and +	0.2076 (0.0437)
Shoot nitrogen concentration (mg g^{-1})							
p=0.084	I	-	19.01 (3.98)	p=0.004	U	- and +	17.03 (3.87)
	I	post	20.47 (6.5)		I	- and +	21.02 (5.54)
	I	pre	23.59 (5.18)				
Root + Nodule nitrogen (mg)							
ns				p=0.084	U	- and +	2.57 (0.65)
					I	- and +	2.934 (0.817)

Pea (Parafield) / Imazethapyr							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Root + Nodule dry weight (g)							
ns				p=0.006	U	- and +	0.1878 (0.040)
					I	- and +	0.1644 (0.0325)
Root + Nodule nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	13.79 (2.63)
					I	- and +	17.91 (3.99)
Root : Shoot dry weight ratio							
ns				p<0.001	U	- and +	1.107 (0.280)
					I	- and +	0.799 (0.207)
Root : Shoot nitrogen ratio							
ns				p=0.013	U	- and +	0.950 (0.432)
					I	- and +	0.727 (0.281)
Nodule dry weight (g)			ns	na			
Nodule number				na			
p=0.003	I	-	18.4 (8.1)				
	I	post	24.2 (11.2)				
	I	pre	12.4 (6.6)				
Dry weight per nodule (mg $nodule^{-1}$)			ns	na			
Nodule dry weight per unit root dry weight (g g^{-1})			ns	na			
Nodule number per unit root dry weight (g^{-1})				na			
Number of nodules per unit root dry weight (g^{-1})							
p=0.007	I	-	108.8 (34.95)	na			
	I	post	158.8 (90.6)				
	I	pre	79.7 (37.9)				
Amount of nitrogen fixed (mg) ns				na			
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			ns	na			
Nitrogen fixed per nodule (mg $nodule^{-1}$) ns				na			

3.3.6 Faba Bean / Imazethapyr (Table 3.9)

There were no main effects of the PSPE application of imazethapyr to faba bean plants. However some rhizobia pre-exposure effects were observed. Pre-exposure of rhizobia to herbicide resulted in a reduction in total plant nitrogen concentration (p=0.01) from 22.1 to 20.9 mg g^{-1}. This was due in particular to a reduction in below ground nitrogen concentration (p=0.005) from 24.1 to 19.5 mg g^{-1} caused by pre-exposure of rhizobia to herbicide and can be attributed to a reduction in below ground nitrogen (p=0.058) rather than a decrease in root dry weight. There was a possible rhizobia pre-exposure by plant

applied herbicide interaction (p=0.052), in which herbicide treated plants inoculated with pre-exposed rhizobia had a particularly low below ground N concentration, whereas the equivalent plants inoculated with unexposed rhizobia had higher below ground and nitrogen concentrations than the unsprayed controls of each rhizobia treatment. A similar trend was found for total plant nitrogen concentrations (p=0.054). There were no significant treatment effects on nodulation or nitrogen fixation.

Table 3.9 Treatment effects and interactions for the bean / imazethapyr experiment. Where significant (p<0.05) treatment means are given, standard deviations are presented are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Faba bean (Fiesta) / Imazethapyr							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
ns				p<0.001	U	- and +	13.76 (2.22)
					I	- and +	27.92 (5.58)
Total plant dry weight (g)							
ns				p<0.001	U	- and +	1.070 (0.160)
					I	- and +	1.364 (0.260)
Total plant nitrogen concentration (mg g^{-1})							
p=0.01	-	- and +	22.10 (2.68)	p=0.005	O	- and +	13.06 (2.42)
	+	- and +	20.87 (2.49)		-	- and +	22.10 (2.68)
p=0.076	-	-	21.38		+	- and +	19.15 (2.49)
	-	+	22.82	p=0.054	O	-	12.83 (2.89)
	+	-	20.39	lsd 2.786	O	+	13.29 (2.04)
	+	+	17.93		-	-	21.38 (2.17)
					-	+	22.82 (3.11)
					+	-	20.39 (2.22)
					+	+	17.93 (2.25)
Shoot nitrogen (mg)							
ns				p<0.001	U	- and +	6.10 (1.60)
					I	- and +	15.12 (3.92)
Shoot dry weight (g)							
ns				p=0.001	U	- and +	0.541 (0.152)
					I	- and +	0.767 (0.209)
Shoot nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	11.71 (2.91)
					I	- and +	20.09 (3.69)
Root + Nodule nitrogen (mg)							
p=0.058	-	- and +	13.67 (2.43)	p=0.027	O	- and +	7.66 (1.34)
	+	- and +	11.92 (2.36)		-	- and +	13.67 (2.43)

Faba bean (Fiesta) / Imazethapyr							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
					+	- and +	11.92 (2.54)
Root + Nodule dry weight (g)							
ns				p=0.062	U	- and +	0.529 (0.068)
					I	- and +	0.597 (0.110)
Root + Nodule nitrogen concentration (mg g^{-1})							
p=0.005	-	- and +	24.11 (4.12)	p=0.001	O	- and +	14.62 (2.69)
	+	- and +	19.48 (3.61)		-	- and +	24.11 (4.12)
p=0.092	-	-	22.69		+	- and +	19.48 (3.61)
	-	+	25.52	p=0.052	O	-	15.14 (3.00)
	+	-	20.66	lsd 3.708	O	+	14.09 (2.45)
	+	+	18.31		-	-	22.69 (3.84)
					-	+	25.52 (4.18)
					+	-	20.66 (3.03)
					+	+	18.31 (3.98)
Root : Shoot dry weight ratio							
p=0.077	I	-	0.885 (0.289)	p=0.007	U	- and +	1.058 (0.349)
	I	+	0.709 (0.154)		I	- and +	0.797 (0.244)
Root : Shoot nitrogen ratio							
p=0.089	I	-	0.948 (0.237)	p<0.001	U	- and +	1.325 (0.391)
	I	+	0.807 (0.155)		I	- and +	0.878 (0.209)
				p=0.027	U and I	-	1.121 (0.397)
					U and I	+	0.933 (0.275)
Nodule dry weight (g)			ns	na			
Nodule number			ns	na			
Dry weight per nodule (g $nodule^{-1}$)			ns	na			
Nodule number per unit root dry weight (g^{-1})			ns	na			
Nodule dry weight per unit root dry weight (g g^{-1})			ns	na			
Amount of nitrogen fixed (mg)			ns	na			
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			ns	na			
Nitrogen fixed per nodule (mg $nodule^{-1}$)			ns	na			

3.3.7 Vetch / Flumetsulam (Table 3.10)

Herbicide labels for vetch now specify a single variety (Popany) for which flumetsulam is recommended for application in-crop. However it has been common practice for farmers to apply flumetsulam to other vetch varieties (D. Roget, personal communication).

Flumetsulam caused obvious symptoms including purple fringes to leaves within 3 days of herbicide application. Main effects of plant applied herbicide on biomass, particularly shoot dry weight were observed. Whereas shoot dry weight in herbicide treated plants declined by 31% compared with controls, shoot nitrogen decreased by only 21% associated with an increase in shoot nitrogen concentration of 14%. There was a reduction in root dry weight ($p=0.024$) caused by plant applied herbicide, along with a small but not statistically significant decrease in the amount of nitrogen in below ground organs.

For this experiment, nodules were sorted into viable and non-viable, based on their appearance (pink or non-pink). Flumetsulam application increased the total number of nodules by 19%. However, this was due to a three-fold increase in the number of non-pink nodules. The number of pink nodules declined by 85%. Since herbicide was applied beyond the three leaf stage, it is likely that the herbicide made existing nodules non-viable and/or formation of additional ineffective nodules occurred. Plant applied herbicide reduced the dry weight per nodule by 29%, consistent with the large number of small, ineffective nodules on these plants. While there was no significant difference in the dry weight of nodules per unit root dry weight, there was an increase in the number of nodules per unit root dry weight with herbicide application, as would be expected from the nodule number data explained above. These results imply that the initiation of nodules was not impeded, but the development of nodules was compromised, or existing nodules became ineffective due to herbicide application to the plant.

The amount of nitrogen fixed by vetch plants was 34% lower when flumetsulam was applied. There was a 45% decrease in the amount of nitrogen fixed per nodule, as would be expected from the increase in the number of ineffective nodules and the decrease in the number of pink nodules observed on plants to which herbicide was applied.

There were no rhizobia pre-exposure effects measured in this experiment.

Table 3.10 Treatment effects and interactions for the vetch / flumetsulam experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Vetch (Popany) / Flumetsulam							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)							
p=0.02	I	-	4.92 (1.09)	p<0.001	U	- and +	3.11 (0.42)
	I	+	4.17 (0.60)		I	- and +	4.53 (0.97)
				p=0.015	U and I	-	4.35 (1.23)
					U and I	+	3.76 (0.75)
Total plant dry weight (g)							
p<0.001	I	-	0.1819 (0.0356)	p<0.001	U and I	-	0.1788 (0.0357)
	I	+	0.1437 (0.0220)		U and I	+	0.1448 (0.0211)
Total plant nitrogen concentration (mg g^{-1})							
p=0.036	I	-	26.98 (2.30)	p<0.001	U	- and +	19.92 (3.85)
	I	+	29.11 (2.38)		I	- and +	28.01 (2.54)
				p=0.048	U and I	-	24.36 (4.57)
					U and I	+	26.28 (5.2)
Shoot nitrogen (mg)							
p=0.01	I	-	2.71 (0.735)	p<0.001	U	- and +	1.343 (0.347)
	I	+	2.13 (0.375)		I	- and +	2.406 (0.658)
				p=0.017	U and I	-	2.261 (0.893)
					U and I	+	1.843 (0.537)
Shoot dry weight (g)							
p<0.001	I	-	0.0947 (0.0268)	p=0.024	U	- and +	0.0661 (0.0164)
	I	+	0.0651 (0.0135)		I	- and +	0.0796 (0.0262)
				p<0.001	U and I	-	0.0871 (0.0264)
					U and I	+	0.0631 (0.0128)
Shoot nitrogen concentration (mg g^{-1})							
p=0.008	I	-	28.87 (3.24)	p<0.001	U	- and +	20.93 (5.87)
	I	+	32.96 (3.62)		I	- and +	30.85 (3.96)
				p=0.015	U and I	-	25.78 (5.77)
					U and I	+	29.3 (7.20)
Root + Nodule nitrogen (mg)							
				p=0.002	U	- and +	1.768 (0.335)
					I	- and +	2.124 (0.447)
				p=0.077	U and I	-	2.094 (0.503)
					U and I	+	1.917 (0.346)
Root + Nodule dry weight (g)							
ns				p=0.02	U	- and +	0.0941 (0.022)
					I	- and +	0.0830 (0.017)
				p=0.024	U and I	-	0.0917 (0.022)
					U and I	+	0.0817 (0.015)

Vetch (Popany) / Flumetsulam							
plant herbicide * rhizobia pre-treatment				**plant herbicide*inoculation/rhizobia pre-treatment**			
ANOVA 1	**Rhizobia treatment**	**Herbicide treatment**	**Mean (SD)**	**ANOVA 2**	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Root + Nodule nitrogen concentration (mg g^{-1})							
ns				p<0.001	U	- and +	19.1 (2.15)
					I	- and +	25.61 (1.88)
Root : Shoot dry weight ratio							
p=0.004	I	-	0.943 (0.273)	p<0.001	U	- and +	1.471 (0.415)
	I	+	1.188 (0.179)		I	- and +	1.073 (0.261)
				p=0.019	U and I	-	1.107 (0.381)
					U and I	+	1.304 (0.345)
Root : Shoot nitrogen ratio							
p=0.065	I	-	0.846 (0.218)	p<0.001	U	- and +	1.408 (0.415)
	I	+	0.971 (0.158)		I	- and +	0.919 (0.199)
p=0.093	-	-	0.927 (0.199)				
lsd 0.190	-	+	0.939 (0.125)				
	+	-	0.765 (0.219)				
	+	+	1.003 (0.187)				
Nodule dry weight (g)			ns	na			
Nodule number				na			
p=0.011	I	-	39.6 (13.0)				
	I	+	47.2 (10.8)				
Pink nodule number				na			
p<0.001	I	-	29.2 (17.8)				
	I	+	4.3 (6.0)				
Dry weight per nodule (mg $nodule^{-1}$)				na			
p=0.004	I	-	0.0781 (0.0215)				
	I	+	0.0557 (0.0153)				
Nodule dry weight per unit root dry weight (g g^{-1})				na			
Nodule number per unit root dry weight (g^{-1})				na			
p=0.002	I	-	487 (177)				
	I	+	631 (134)				
Pink nodule number per unit root dry weight (g^{-1})							
p<0.001	I	-	340 (213)	na			
	I	+	54 (95)				
Amount of nitrogen fixed (mg)				na			
p=0.033	I	-	1.7 (1.05)				
	I	+	1.12 (0.76)				
Nitrogen fixed per unit nodule dry weight (mg g^{-1})							
p=0.096	I	-	545 (263)	na			
	I	+	408 (284)				
Nitrogen fixed per nodule (mg $nodule^{-1}$)				na			
p=0.004	I	-	0.0428 (0.0243)				
	I	+	0.0236 (0.0154)				

3.4 Summary of results

The experiments described in this chapter confirm that group B herbicides have the potential to reduce nitrogen fixation by grain legumes. Generally where large reductions in nitrogen fixation occurred there was also obvious injury or growth inhibition to the plant caused by plant-applied herbicide. For example the PSPE application of imazethapyr clearly damaged the roots of chickpea plants and reduced the amount of N_2 fixed by 60%. The roots were severely stunted, with shorter lateral roots and 'arrow tips' compared to controls (Figure 3.1). Visual symptoms (discolouration of outer edges of leaves) were obvious on vetch plants 3 days after foliar application of flumetsulam. Growth of shoots of pea plants was stunted (Figure 3.2) and new leaves pale in colour after foliar application of imazamox, with interveinal chlorosis evident on the youngest leaves.

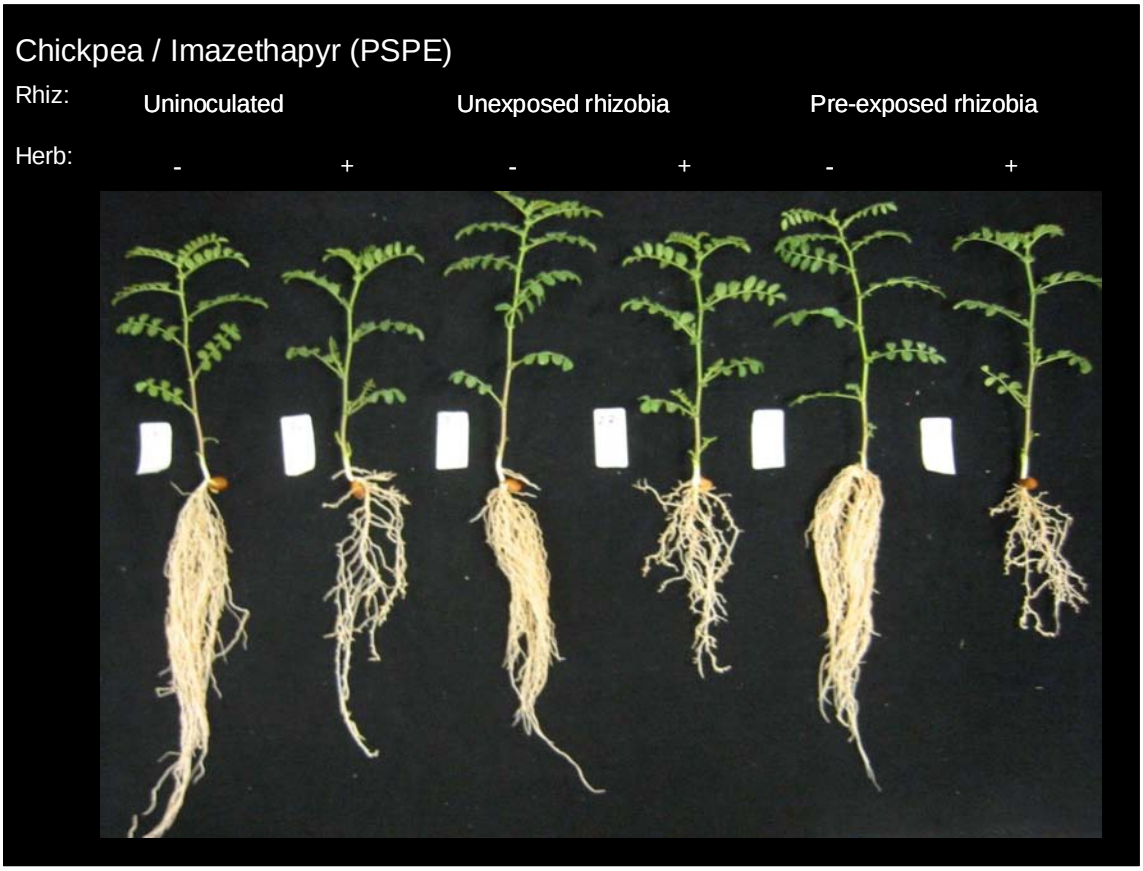

Figure 3.1 Chickpea / imazethapyr experiment showing lower root mass, stunted lateral roots and deformed meristematic region of roots in herbicide treated plants

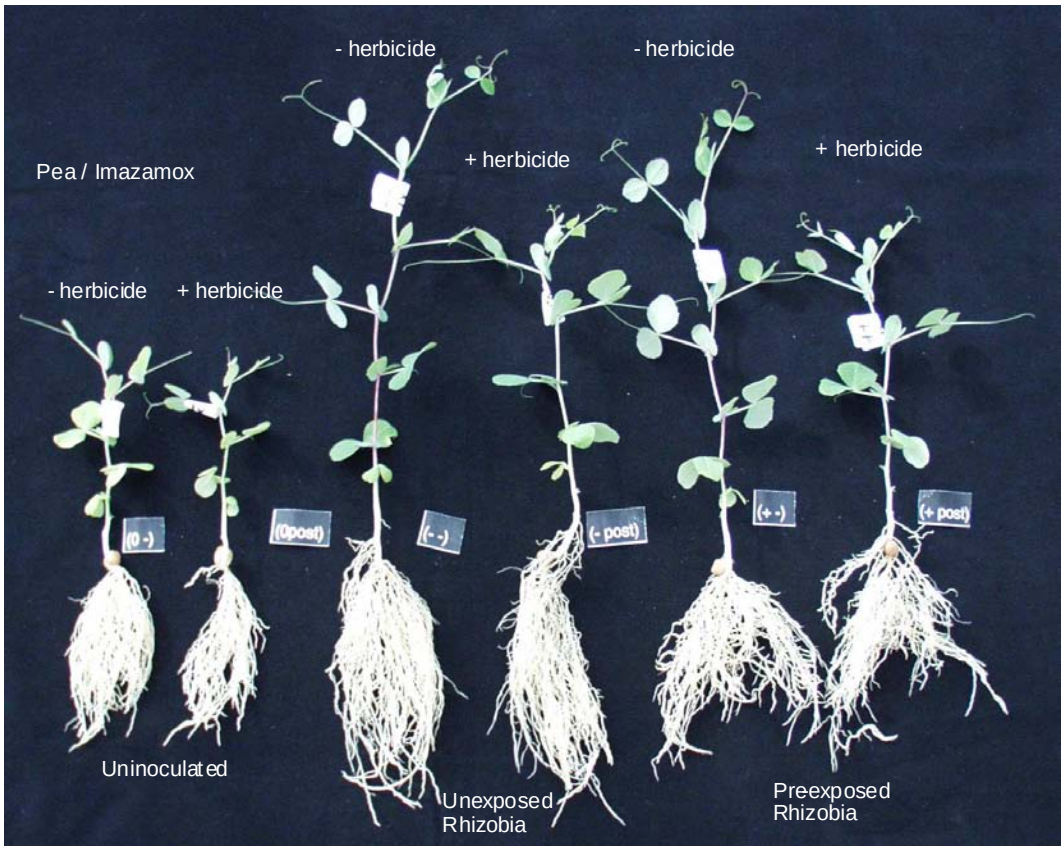

Figure 3.2 Pea / imazamox experiment showing reductions in shoot biomass.

Whilst in some experiments reductions in N_2 fixation coincided with clear symptoms on the plant, even where symptoms and effects on plant growth were not obvious, reduced nitrogen fixation could still be detected. For example in the pea / flumetsulam experiment there was little growth inhibition due to herbicide application, however there was a 31% reduction in the amount of nitrogen fixed and yellowing of leaves on some plants could be seen (Figure 3.3).

Figure 3.3 Leaf yellowing of pea plants induced by flumetsulam application (right) compared with a control plant (left).

The PSPE application of imazethapyr to chickpea caused obvious damage to roots but the number of nodules expressed on a root dry weight basis was not significantly affected. In that experiment nodule dry weight declined more than nodule number, indicating that imazethapyr applied PSPE caused a greater inhibition to the development of nodules than nodule initiation. A decrease in the nodule dry weight per unit root dry weight also indicated that nodule development was more sensitive to herbicide than root growth. The pea / imazethapyr experiment allowed the direct comparison of PSPE application with post emergent application at the 4 node stage. The PSPE application reduced the number of nodules compared to controls, while the post-emergent application, which would have occurred after nodulation had commenced, increased the number of nodules.

The impacts of group B herbicides on root growth and nodulation were not restricted to PSPE herbicide applications. Effects on roots and nodules were also observed when herbicide was applied to the shoots. Flumetsulam reduced root plus nodule dry weight

and nitrogen of chickpea plants, despite being applied to the shoots. Of particular interest was the nodulation data in the vetch / flumetsulam experiment in which nodule viability was visually assessed. The increase in the total number of nodules per vetch plant due to flumetsulam application was due to an increase in the number of small, white nodules that can be assumed to be non-fixing. There was actually a decrease in the number of pink nodules. Since flumetsulam was applied as a post-emergence application, it was expected that by the time of herbicide application a substantial number of effective nodules had already formed. It was therefore likely that the herbicide caused many of the existing nodules to become ineffective. The formation of additional nodules might have been a response to the loss of effective existing nodules or a more direct effect of the herbicide on meristems. It is apparent that the additional nodules that formed also became ineffective.

In the *in vitro* experiments in this work, the group B herbicides tested did not inhibit the growth of rhizobia at concentrations relevant to field application. Pre-exposure of rhizobia to herbicide was found to affect some of the variates in three of the experiments described here, but in a minor and unpredictable manner. The effects were not always disruptive. In the chickpea / flumetsulam experiment, unsprayed plants inoculated with pre-exposed rhizobia had higher amounts of total plant nitrogen than those inoculated with unexposed rhizobia. In the pea / imazamox experiment, pre-exposure of rhizobia to herbicide prevented a decline in plant nitrogen that occurred when herbicide was applied to plants inoculated with unexposed rhizobia. However, in the faba bean / imazethapyr experiment pre-exposure of rhizobia to herbicide reduced the amount of nitrogen in roots and nodules, linked to a reduction in the nitrogen concentration in these organs. In contrast, the fact that herbicide impacts were observed on uninoculated plants indicates that plant growth and function was an important susceptibility in the symbiosis.

The experiments presented here clearly show that group B herbicides can inhibit nitrogen fixation by grain legumes. Reductions in nodulation and nitrogen fixation of up to 60% were observed with herbicide application at label rates. However, there was no consistent response to the herbicides tested amongst the legumes examined. Even when symptoms were not pronounced, the application of herbicide as per label recommendations reduced nitrogen fixation. There were also cases where visual

symptoms of herbicide application were noted and reductions in nodulation measured, but no significant reduction in the amount of nitrogen fixed occurred. An increased understanding of the mechanisms involved is therefore required before general recommendations to alter herbicide practices can be made. Thus the major practical outcome from the work described in this chapter is the confirmation that group B herbicides can compromise nitrogen fixation by grain legumes.

Chapter 4 The impact of 'in-crop' group B herbicides on the growth, nodulation and nitrogen fixation of pasture legumes

CHAPTER 4 .... THE IMPACT OF 'IN-CROP' GROUP B HERBICIDES ON THE GROWTH, NODULATION AND NITROGEN FIXATION OF PASTURE LEGUMES104

4.1 Introduction

Legumes in pastures are primarily valued for their ability to biologically fix nitrogen in symbiosis with rhizobia (Charman and Ballard, 2004). Legume based pastures are estimated to occur on 12.7 of the 13.6 million hectares of improved pastures in southern Australia (ABS 1998 after Peoples and Baldock 2001). When reliant on biological fixation of N_2, such pastures fix between 20 and 25 kg of shoot nitrogen for each tonne of legume shoot dry matter produced (Peoples and Baldock, 2001). Contributions to the nitrogen balance are likely to be further enhanced by root and nodule nitrogen which

account for up to 50% of the total N_2 fixed by legumes (McNeill and Fillery, 2008). Pasture legumes can be exposed to group B herbicides either from application directly to the pasture for post emergent control of broadleaf weeds, or from herbicide residues in the soil from previous applications. This chapter aims to screen the recommended 'in crop' group B herbicides (flumetsulam, imazamox and imazethapyr) for impacts on the growth, nodulation and nitrogen fixation of pasture legumes commonly grown in southern Australia (lucerne, burr medic, subterranean clover, balansa clover).

4.2 Materials and Methods

A similar experimental design to the screening of grain legumes (Chapter 3) was used. Experiments were set up in a factorial design, with three inoculation treatments and two plant applied herbicide treatments:

3 inoculation treatments -

- Uninoculated
- Washed rhizobia (denoted '-')
- Rhizobia grown in the presence of herbicide and washed (denoted '+')

The third inoculation treatment (rhizobia grown in the presence of herbicide and washed) was not included in the subterranean and balansa clover experiments due to difficulties synchronising culture growth after failure of temperature control during incubation.

2 plant-applied herbicide treatments -

- Herbicide applied at the rate, method and stage recommended on the herbicide label (denoted '+')
- No herbicide at all (denoted '-')

Each experiment had between 5 and 7 blocks, depending on the success of plant germination for each experiment. The various combinations of pasture legume species and herbicides examined in this work are summarised in Table 4.1

Table 4.1 Inoculum strains, and application rates for the herbicides as commercial formulations (imazethapyr 70% w/w as Spinnaker herbicide, flumetsulam 80% w/w as Broadstrike herbicide and imazamox 70% w/w as Raptor herbicide) and active ingredient in parentheses. BS1000 is a non-ionic surfactant. The plant growth stage for herbicide application and harvest (days after application) are also given.

Legume	Inoculum	Herbicide	Rate (g ha^{-1})	Additive	Growth Stage	Harvest
Lucerne *Medicago sativa* Super 7	AL RRI128	Imazamox	50 (45 ai)		3rd trifoliate on	11 days
		Flumetsulam	25 (20 ai)	BS1000	2-3 trifoliate on	15 days
		Imazethapyr	140 (98 ai)	BS1000	2nd trifoliate on	8 days
Burr medic *Medicago polymorpha* Santiago	AM WSM1115	Flumetsulam	25 (20 ai)	BS1000	2-3 trifoliate on	14 days
		Imazamox	50 (45 ai)		3rd trifoliate on	10 days
Subterranean clover *Trifolium subterraneum* York	C WSM409	Imazethapyr	50 (45 ai)		3 leaf on	13 days
		Imazamox	50 (45 ai)		3rd trifoliate on	14 days
		Flumetsulam	25 (20 ai)	BS1000	2-3 trifoliate on	15 days
Balansa clover *Trifolium michelianum* Frontier	C WSM409	Imazamox	50 (45 ai)		3rd trifoliate on	10 days
		Flumetsulam	25 (20 ai)	BS1000	3rd trifoliate on	8 days

Pasture legumes were grown in 500 ml re-usable Sarstedt culture jars with lids through which 2 holes were drilled to allow seedling growth and watering. The lids reduced evaporation, prevented rhizobial contamination and minimised potential herbicide dose to the roots since no surface spray could be washed into the sand.

All of the pasture legume plants were pre-germinated. Seeds were surface sterilised for 1 minute in 70% ethanol, followed by 5 minutes in a 3% sodium hypochlorite solution. Seeds were then rinsed 8 times in sterile water, and placed on 1% water agar plates. Plates were sealed with parafilm, wrapped in foil and stored for 24 hours at 4°C to synchronise subsequent germination. Plates were then incubated upside down at 25°C to encourage the radicles to grow away from the agar and allow easy transfer to the jars. Germinated seedlings were transferred to 500 ml jars filled with 810 g of N40 sand that

had been autoclaved previously. Temperatures in the glasshouse were maintained at 25°C during the day and 15°C at night.

Previous work had identified that the pasture species grew better with McKnight's than in Tang's nutrient solution. Whilst McKnight's was not amenable to adjusting the pH, this was deemed not to be an issue with the pasture experiments, since there was no requirement to apply herbicide as a post-sowing pre-emergence application and therefore a high pH was not required to prevent hydrolysis and maintain the availability of the herbicide as per field conditions. Full strength nitrogen free McKnight's was composed of $FeCl_3$ (0.87 mM), KH_2PO_4 (1.47 mM), $CaSO_4.2H_2O$ (8.72 mM), $MgSO_4.7H_2O$ (0.813 mM), KCl (4.054 mM), H_3BO_3 (0.046 mM), $MnSO_4.H_2O$ (9 μM), $ZnSO_4.7H_2O$ (0.776 μM), $CuSO_4.5H_2O$ (0.317 μM), $H_2MoO_4.H_2O$ (0.495 μM). Seedlings were planted into jars which had been watered to weight with sterile ¼ strength McKnight's nutrient solution added aseptically using a sterile dispenser to inject it through the holes in the jar lids. A flame sterilised metallic prong was used to form two small holes in the sand, into which the radicles of germinated seedlings were carefully placed. After establishment (approximately 2 days), seedlings were thinned to one per jar with the aim of ensuring uniform plant size within each block.

Inoculum was prepared and growth curves for each Rhizobium strain were obtained as described in Chapter 3.

The amount of herbicide added was calculated on a surface area and volume basis (Table 4.2). Twenty-four to 48 hours after seeding all broth cultures from a single starter culture, the optical densities at 500 *n*m were measured. Dilution series were made in PBS to determine the number of cfu in the culture by drop plate counts.

To inoculate each treatment with an equivalent number of rhizobia, the number of cfu in each culture was estimated from the previously determined relationships between optical density of cultures at 500 *n*m and the cfu per ml of culture. A washing procedure was used to remove any residual herbicide from the inoculum described in Chapter 3.

Table 4.2 Recommended application rates of herbicide as the commercial formulation (imazethapyr 70% w/w as Spinnaker herbicide, flumetsulam 80% w/w as Broadstrike herbicide and imazamox 70% w/w as Raptor herbicide) and the amounts of commercial formulation used in the rhizobia cultures

Herbicide	Label rate for commercial formulation (g ha^{-1})	Amount per litre (µg)	Amount of commercial formulation per 50 ml culture (µg)	Amount of active ingredient per 50ml culture (µg)
Imazethapyr	50	50	2.5	1.75
Imazethapyr	140	70	3.5	2.45
Flumetsulam	25	25	1.25	1
Imazamox	50	50	2.5	1.75

A 1 ml volume of a phosphate buffered saline suspension of rhizobia to give 10^3 cells per gram of sand was pipetted onto the sand surrounding the transferred seedlings. For most experiments this was done on the same day as the seedlings were transferred.

Post inoculation jars were watered aseptically as needed through the vacant hole in the lid to a pre-determined weight indicative of field capacity.

Herbicide was applied to plants at the recommended growth stage at label rates (see Table 4.1) using the spray cabinet as described in Chapter 3. Surfactants were added where recommended. Lids remained on jars, which prevented herbicide entering the sand. This likely reduced the dose of herbicide compared to what may be received in the field. Control plants were unsprayed.

Experiments were harvested between 7 and 14 days after herbicide application, as described in Chapter 3. Shoots were excised from roots. Roots were washed of sand, and nodules removed and counted. Shoots, roots and nodules were dried to constant mass at 55 to 60°C (at least 24 hours) and weighed separately. Shoots and roots plus nodule samples were digested whole to determine the nitrogen content by the modified Kjeldahl method described in Chapter 3. An index of the relative amount of root and shoot biomass and nitrogen was calculated by dividing the root plus nodule dry weight

by the shoot dry weight. The amount of nitrogen fixed was calculated as presented in Chapter 3.

Due to the small size of pasture legume seed, there was little nitrogen in reserve for initial growth of seedlings. Therefore the usefulness of uninoculated plants in these experiments was limited to checking for contaminating rhizobia and for calculating the amount of nitrogen fixed, rather than detecting effects of the herbicide on the plant independent of symbiotic nitrogen fixation. Therefore, these experiments were analysed by ANOVA of inoculated plants only, with a factorial design of plant-applied herbicide by rhizobia pre-treatment. For the subterranean and balansa clover experiments, only the herbicide treatment was tested statistically. All statistical analyses were completed using Genstat 5 (Release 4.1 1997 VSN International Ltd: Hemel Hempstead, UK).

4.3 Results

There were no significant effects of any of the herbicides on the *in vitro* growth of the rhizobia strains as measured by the optical density of cultures and subsequently by drop plate counts.

A variety of responses were observed in the glasshouse experiments, ranging from no detectable differences in the measured variates, to clear differences in growth, nodulation and nitrogen fixation. A summary of significant treatment effects are shown in Table 4.3.

Table 4.3 Summary of significant ($p<0.05$) treatment effects of herbicide application (H) or pre-exposure of rhizobia (R). Main effects of inoculation not shown.

Treatments	Lucerne Imazamox H*R	Lucerne Flumetsulam H*R	Lucerne Imazethapyr H*R	Burr medic Flumetsulam H*R	Burr medic Imazamox H*R	Subterranean clover Imazethapyr H	Subterranean clover Imazamox H	Subterranean clover Flumetsulam H	Balansa clover Imazamox H	Balansa clover Flumetsulam H
Total plant nitrogen (mg)	H	R						H	H	H
Total plant dry weight (g)	H	R						H	H	H
Total plant nitrogen concentration (mg g^{-1})	H*R	H		R					H	H
Shoot nitrogen (mg)	H	R						H	H	H
Shoot dry weight (g)	H	R						H	H	H
Shoot nitrogen concentration (mg g^{-1})	H*R	H							H	H
Root + Nodule nitrogen (mg)	H	R		H*R					H	
Root + Nodule dry weight (g)	H	R	R	H*R					H	
Root + Nodule nitrogen concentration (mg g^{-1})	H*R	R		H*R						
Root : Shoot biomass ratio	H	H*R			R			H		
Root : Shoot nitrogen ratio	H	H*R						H		
Nodule dry weight (g)	H		H*R		H*R			H	H	H
Nodule number	H		H	H*R	H					
Pink nodule dry weight	na		H*R	R	H*R	na	na	na	na	na
Pink nodule number	H	R		H*R	H			H		
Dry weight per nodule (g $nodule^{-1}$)			H*R	R						H
Dry weight per pink nodule (g $nodule^{-1}$)	na		H*R		R	na	na	na	na	na
Nodule dry weight per unit root dry weight (g g^{-1})	H*R									
Nodule number per unit root dry weight (g^{-1})										
Pink nodule dry weight per unit root dry weight (g g^{-1})	na			R		na	na	na	na	na
Pink nodule number per unit root dry weight (g^{-1})		R		R						
Amount of nitrogen fixed (mg)	H	R						H	H	H
Nitrogen fixed per unit nodule dry weight (mg g^{-1})	H	H			H R					
Nitrogen fixed per nodule (mg $nodule^{-1}$)	H		H	H*R	H					
Nitrogen fixed per unit pink nodule dry weight (mg g^{-1})	na			R	H	na	na	na	na	na
Nitrogen fixed per pink nodule (mg $nodule^{-1}$)				H*R	H					

The major findings for each pasture legume experiment are described below. Data from individual experiments are presented in tables at the end of each section. Significant differences ($p<0.05$) are presented, along with trends where they convey useful information. Variates where there were significant treatment effects ($p<0.05$) are given, along with trends where they convey useful information.

4.3.1 Lucerne (Super 7) / Imazamox (Table 4.4)

Plant-applied herbicide affected most variates measured in this experiment, indicating that Super 7 lucerne lacks tolerance to imazamox even at recommended application rate of 50 g ha^{-1}. Plant applied herbicide reduced shoot dry weight by 23% and root plus nodule dry weight by 43%. There was also a main effect of plant-applied herbicide on the amount of nitrogen in shoots and roots plus nodules, with a 16% and 33% reduction respectively compared to controls.

Nitrogen concentrations of shoots and recovered below ground biomass were affected by an interaction between plant-applied herbicide and pre-exposure of rhizobia. For unsprayed plants, the shoot nitrogen concentration for plants inoculated with unexposed and pre-exposed rhizobia was similar at 31.0 and 30.7 mg g^{-1} respectively. However when imazamox was applied to plants, the nitrogen concentration of shoots increased to 37.6 mg g^{-1} for plants inoculated with unexposed rhizobia, but was unchanged at 30.5 mg g^{-1} for plants inoculated with pre-exposed rhizobia. The below ground nitrogen concentration was affected by the treatments in a similar way, with the exception that the herbicide application to plants inoculated with pre-exposed rhizobia had a significantly higher below ground nitrogen concentration than unsprayed plants inoculated with unexposed rhizobia.

Plant-applied herbicide caused a larger decrease in nodulation more than it did shoot dry weight. Herbicide application reduced the number of pink nodules per plant from 44 to 20, and the total nodule dry weight decreased from 7.7 mg to 3.5 mg. When the data was log transformed, the dry weight per nodule was found to increase due to pre-exposure of rhizobia to herbicide prior to inoculation ($p=0.018$). There were no significant differences between treatments for nodulation on a root mass basis, though there was a trend in which control plants inoculated with pre-exposed rhizobia had a

higher nodule dry weight per unit root dry weight than the other herbicide by rhizobia treatment combinations (p=0.061).

Plant-applied herbicide was calculated to have reduced the amount of nitrogen fixed by 25% (p=0.006). However the amount of nitrogen fixed per unit nodule mass increased by 53% (p=0.017) with herbicide application. There was also a trend (p=0.057) in which pre-exposure of rhizobia to herbicide increased the amount of nitrogen fixed per nodule by 31%.

Table 4.4 Treatment effects and interactions for the lucerne / imazamox experiment. Where significant (p<0.05) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Lucerne (Super 7) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			
p=0.008	I	-	4.96 (1.10)
	I	+	3.82 (1.82)
Total plant dry weight (g)			
p<0.001	I	-	0.1769 (0.0502)
	I	+	0.1205 (0.0667)
Total plant nitrogen concentration (mg g^{-1})			
p=0.006	-	-	27.73 **a**
LSD 2.9	-	+	35.19 **b**
	+	-	28.50 **a**
	+	+	29.69 **a**
Shoot nitrogen (mg)			
p=0.057	I	-	3 (0.72)
	I	+	2.51 (1.16)
Shoot dry weight (g)			
p=0.002	I	-	0.0980 (0.03)
	I	+	0.0753 (0.04)

Lucerne (Super 7) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nodule dry weight (mg)			
p<0.001	I	-	7.68 (3.2)
	I	+	3.51 (2.4)
Nodule number			
p=0.011	I	-	48.1 (31)
	I	+	24.4 (14)
Pink nodule number			
p=0.013	I	-	43.7 (32)
	I	+	19.6 (9)
Dry weight per nodule (mg nodule^{-1}) ns			
Nodule dry weight per unit root dry weight (g g^{-1})			
p=0.061	-	-	0.0886
LSD 0.055	-	+	0.1040
	+	-	0.1323
	+	+	0.0742
Nodule number per unit root dry weight (g^{-1}) ns			
Pink nodule number per unit root dry weight (g^{-1}) ns			

Lucerne (Super 7) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Shoot nitrogen concentration (mg g^{-1})			
p=0.028	-	-	31.04 **a**
LSD 4.18	-	+	37.61 **b**
	+	-	30.71 **a**
	+	+	30.49 **a**
Root + nodule nitrogen (mg)			
p<0.001	I	-	1.957 (0.514)
	I	+	1.312 (0.691)
Root + nodule dry weight (g)			
p<0.001	I	-	0.0789 (0.022)
	I	+	0.0452 (0.027)
Root + nodule nitrogen concentration (mg g^{-1})			
p=0.016	-	-	24.01 **a**
LSD 2.63	-	+	31.33 **c**
	+	-	25.84 **ab**
	+	+	28.39 **b**
Root : Shoot dry weight ratio			
p=0.002	I	-	0.741 (0.140)
	I	+	0.556 (0.123)
Root : Shoot nitrogen ratio			
p=0.006	I	-	0.657 (0.088)
	I	+	0.532 (0.119)

Lucerne (Super 7) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nitrogen fixed (mg)			
p=0.006	I	-	4.64 (1.17)
	I	+	3.48 (1.77)
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			
p=0.017	I	-	744 (374)
	I	+	1136 (488)
Nitrogen fixed per nodule (mg $nodule^{-1}$)			
p=0.079	I	-	0.111 (0.107)
	I	+	0.173 (0.073)
Nitrogen fixed per pink nodule (mg $nodule^{-1}$)			
p=0.057	-	- and+	0.133 (0.06)
	+	- and +	0.193 (0.11)

4.3.2 Lucerne / Flumetsulam (Table 4.5)

In contrast to the lucerne / imazamox experiment, plant-applied flumetsulam did not significantly alter the biomass of lucerne plants. However plant-applied herbicide increased the concentration of nitrogen in shoots (p=0.02) by 9.6% and possibly reduced the nitrogen concentration of roots plus nodules (p=0.053) by 13.7%. In this experiment, pre-exposure of rhizobia to herbicide increased the dry weight and amount of nitrogen in shoots and roots plus nodules. Consequently, the amount of nitrogen

fixed by plants inoculated with pre-exposed rhizobia increased by 27% compared with plants inoculated with unexposed rhizobia (p=0.009).

Although total nodule dry weight did not change, the number of pink nodules was 35% higher in plants inoculated by pre-exposed rhizobia (p=0.049). Pre-exposure of rhizobia to herbicide reduced the dry weight of pink nodules per unit root dry weight by 34% (p=0.022). However the amount of N_2 fixed per unit of pink nodule dry weight was 27% greater for plants inoculated by pre-exposed rhizobia (p=0.044).

Partitioning of biomass (p=0.021) and nitrogen (p=0.024) between shoots and roots was affected by a herbicide by rhizobia pre-exposure interaction. Plants that were inoculated with unexposed rhizobia and subsequently had flumetsulam applied to shoots had a lower proportion of biomass and nitrogen in the roots compared with the other 3 combinations. Herbicide applied to plants inoculated with pre-exposed rhizobia did not cause a significant shift in the partitioning of biomass or nitrogen.

Some extra uninoculated plants were supplied with nitrogen and the effect of flumetsulam assessed. Only a minor reduction in the amount of nitrogen in roots in particular was detected.

Table 4.5 Treatment effects and interactions for the lucerne / flumetsulam experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Lucerne (Super 7) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			
p=0.01	-	- and +	2.221 (0.402)
	+	- and +	2.731 (0.484)
Total plant dry weight (g)			
p=0.002	-	- and +	0.0620 (0.014)

Lucerne (Super 7) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nodule dry weight (g)			ns
Nodule number			ns
Pink nodule number			
p=0.049	-	- and +	15.3 (9)
	+	- and +	20.7 (9)

Lucerne (Super 7) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
	+	- and +	0.0802 (0.012)
Total plant nitrogen concentration (mg g^{-1})			
p=0.035	I	-	33.6 (2.87)
	I	+	36.82 (3.83)
Shoot nitrogen (mg)			
p=0.014	-	- and +	1.282 (0.219)
	+	- and +	1.498 (0.217)
Shoot dry weight (mg)			
p=0.003	-	- and +	34.83 (6.6)
	+	- and +	41.42 (4.8)
Shoot nitrogen concentration (mg g^{-1})			
p=0.002	I	-	34.93 (1.92)
	I	+	38.3 (3.13)
Root + nodule nitrogen (mg)			
p=0.012	-	- and +	0.939 (0.24)
	+	- and +	1.233 (0.29)
Root + nodule dry weight (g)			
p=0.005	-	- and +	0.0272 (0.009)
	+	- and +	0.0387 (0.008)
Root + nodule nitrogen concentration (mg g^{-1})			
p=0.053	-	- and +	36.6 (7.7)
	+	- and +	31.6 (2.7)
Root : Shoot dry weight ratio			
p=0.021	-	-	0.892 **a**
LSD 0.227	-	+	0.597 **b**
	+	-	0.858 **a**
	+	+	0.953 **a**
Root : Shoot nitrogen ratio			
p=0.024	-	-	0.848 **a**
LSD 0.1323	-	+	0.63 **b**
	+	-	0.816 **a**
	+	+	0.819 **a**

Lucerne (Super 7) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Proportion of nodule dry weight pink			
p=0.026	-	-	0.891 **a**
LSD 0.183	-	+	0.680 **b**
	+	-	0.758 **a**
	+	+	0.849 **a**
Proportion of nodules pink			
p=0.024	-	-	0.672 **ab**
LSD 0.125	-	+	0.626 **b**
	+	-	0.618 **b**
	+	+	0.781 **a**
Dry weight per nodule (mg $nodule^{-1}$) ns			
Nodule mass per unit root dry weight (g g^{-1}) ns			
Nodule number per unit root dry weight (g^{-1}) ns			
Pink nodules per unit root dry weight (g^{-1}) ns			
Pink nodule dry weight per unit root dry weight (g g^{-1})			
p=0.022	-	- and +	0.0411 (0.017)
	+	- and +	0.0270 (0.007)
Nitrogen fixed (mg)			
p=0.009	-	- and +	1.682 (0.42)
	+	- and +	2.142 (0.48)
Nitrogen fixed per unit nodule dry weight (mg g^{-1}) ns			
Nitrogen fixed per unit pink nodule dry weight (mg g^{-1})			
p=0.044	I	-	1823 (641)
	I	+	2318 (780)
Nitrogen fixed per nodule (mg $nodule^{-1}$) ns			

Analysis of uninoculated, N fertilised plants			
Total plant nitrogen (mg)			
p=0.053	N	-	2.098 (0.278)
	N	+	1.813 (0.072)
Root nitrogen (mg)			
p=0.037	N	-	1.107 (0.205)
	N	+	0.919 (0.080)

4.3.3 Lucerne / Imazethapyr (Table 4.6)

There were few significant effects of herbicide or rhizobia pre-exposure treatments in this experiment, though some effects of pre-exposing rhizobia to herbicide were detected. Pre-exposure of rhizobia reduced the total number of nodules per plant from 33 to 26 ($p=0.048$), but there were no significant changes to the number of pink nodules. Pre-exposure of rhizobia to herbicide prior to inoculating appeared to have reduced root plus nodule dry weight (from 45.6 to 41.5 mg per plant, $p=0.092$). Application of imazethapyr to the plant interacted with pre-exposure of rhizobia to affect the dry weight per nodule ($p=0.014$) and dry weight per pink nodule ($p=0.036$) in a similar pattern. Herbicide application did not change the dry weight per nodule for plants inoculated with pre-exposed rhizobia. However for plants inoculated with unexposed rhizobia, the dry weight per nodule decreased with herbicide application to the plant. There were some trends in which herbicide application and pre-exposure of rhizobia to herbicide reduced the nodule dry weight ($p=0.083$) and pink nodule dry weight ($p=0.079$) compared with unsprayed plants inoculated with unexposed rhizobia. Also, the amount of nitrogen fixed per pink nodule appeared to be a third higher for plants inoculated with pre-exposed rhizobia ($p=0.075$).

Table 4.6 Treatment effects and interactions for the lucerne / imazethapyr experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Lucerne (Super 7) / Imazethapyr			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			ns
Total plant dry weight (g)			ns
Total plant nitrogen concentration (mg g^{-1})			ns
Shoot nitrogen (mg)			ns
Shoot dry weight (g)			ns

Lucerne (Super 7) / Imazethapyr			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nodule dry weight per unit root dry weight (g g^{-1})			ns
Nodule number per unit root dry weight (g^{-1})			ns
Pink nodule number per unit root dry weight (g^{-1})			ns
Nitrogen fixed (mg)			ns
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			ns

Lucerne (Super 7) / Imazethapyr			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Shoot nitrogen concentration (mg g^{-1}) ns			
Root + nodule nitrogen (mg) ns			
Root + nodule dry weight (g)			
p=0.092	-	- and +	0.0456 (0.01)
	+	- and +	0.0415 (0.008)
Root + nodule nitrogen concentration (mg g^{-1}) ns			
Root : Shoot dry weight ratio ns			
Root : Shoot nitrogen ratio ns			
Nodule dry weight (mg)			
p=0.083	-	-	2.171
LSD 0.0005	-	+	1.687
	+	-	1.541
	+	+	1.675
Pink nodule dry weight (mg)			
p=0.079	-	-	1.938
LSD 0.0004	-	+	1.487
	+	-	1.393
	+	+	1.512
Nodule number			
p=0.048	-	- and +	33.1 (11)
	+	- and +	26.7 (12)
Pink nodule number ns			
Other nodule number			
p=0.087	-	- and +	9.05 (5.5)
	+	- and +	5.9 (3.9)
Dry weight per nodule (mg nodule^{-1})			
p=0.014	-	-	0.0708 **a**
LSD 0.019	-	+	0.0473 **b**
	+	-	0.0575 **ab**
	+	+	0.069 **a**
Dry weight per pink nodule (mg nodule^{-1})			
p=0.036	-	-	0.0879 **ab**
LSD 0.031	-	+	0.0574 **b**
	+	-	0.072 **ab**
	+	+	0.0886 **a**

Lucerne (Super 7) / Imazethapyr			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Nitrogen fixed per nodule (mg nodule^{-1}) ns			
Nitrogen fixed per nodule (mg nodule^{-1})			
p=0.075	-	- and +	0.0741 (0.027)
	+	- and +	0.0983 (0.042)

Analysis of uninoculated, N fertilised plants			
Root + nodule nitrogen			
p=0.077	N	-	0.91 (0.262)
	N	+	0.82 (0.218)
Root + nodule to shoot nitrogen ratio			
p=0.042	N	-	1.307 (0.376)
	N	+	1.092 (0.328)

4.3.4 Burr medic / Flumetsulam (Table 4.7)

Subtle effects that indicated the potential for flumetsulam to reduce nitrogen fixation by burr medic were observed in this experiment. Pre-exposure of rhizobia to herbicide resulted in reduced total plant nitrogen concentration from 34.9 mg g^{-1} to 32.8 mg g^{-1} (p=0.045). Plants inoculated with pre-exposed rhizobia also had a lower proportion of total nodules that were pink (p=0.015). Some compensation was evident however, since the amount of nitrogen fixed per unit pink nodule dry weight was 36% greater for plants inoculated with pre-exposed rhizobia (p=0.041). Pink (p=0.002) and total nodule number (p=0.05) followed a similar herbicide by rhizobia pre-exposure interaction whereby plant-applied herbicide increased the number of nodules on plants inoculated with unexposed rhizobia, but decreased the number of nodules on plants inoculated with pre-exposed rhizobia. There were no treatment effects on the amount of nitrogen fixed, hence the amount of nitrogen fixed per pink nodule decreased with herbicide application to plants inoculated with unexposed rhizobia, and increased for plants inoculated with pre-exposed rhizobia.

There were no significant effects of flumetsulam on uninoculated plants supplied with nitrogen.

Table 4.7 Treatment effects and interactions for the burr medic / flumetsulam experiment. Where significant (p<0.05) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			ns
Total plant dry weight (g)			ns
Total plant nitrogen concentration (mg g^{-1})			
p=0.045	-	- and +	34.90 (4.37)

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Proportion of nodule dry weight that was pink			
p=0.015	-	- and +	0.907 (0.04)
	+	- and +	0.832 (0.08)
Dry weight per nodule (mg $nodule^{-1}$)			

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
	+	- and +	32.82 (3.77)
Shoot nitrogen (mg)			ns
Shoot dry weight (g)			ns
Shoot nitrogen concentration (mg g^{-1})			ns
Root + nodule nitrogen (mg)			
p=0.077	-	-	1.001
LSD 0.202	-	+	1.104
	+	-	1.071
	+	+	0.921
Root + nodule dry weight (g)			
p=0.059	-	-	0.0226
LSD 0.0078	-	+	0.0284
	+	-	0.0298
	+	+	0.0250
Root + nodule nitrogen concentration (mg g^{-1})			
p=0.096	-	-	48.5
LSD 10.8	-	+	42.1
	+	-	36.4
	+	+	42.8
Root : Shoot dry weight ratio			ns
Root : Shoot nitrogen ratio			ns
Nodule dry weight (g)			ns
Pink nodule dry weight (mg)			
p=0.059	-	- and +	2.69 (1.0)
	+	- and +	2.06 (1.3)
Nodule number			
p=0.05	-	-	41.9 **ab**
LSD 15	-	+	48.3 **ab**
	+	-	52.3 **a**
	+	+	37.5 **b**
Pink nodule number			
p=0.002	-	-	23.1 **bc**
LSD 6	-	+	31.2 **a**
	+	-	27.1 **ab**
	+	+	19.9 **c**

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
p=0.076	-	- and +	0.073 (0.031)
	+	- and +	0.056 (0.018)
Dry weight per pink nodule (mg nodule^{-1})			ns
Nodule dry weight per unit root dry weight (g g^{-1})			ns
Pink nodule dry weight per unit root dry weight (g g^{-1})			
p=0.003	-	- and +	0.1297 (0.05)
	+	- and +	0.0832 (0.03)
Nodule number per unit root dry weight (g^{-1})			
Pink nodules per unit root dry weight (g^{-1})			
p=0.079	-	- and +	1513 (1165)
	+	- and +	1043 (481)
Nitrogen fixed (mg)			ns
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			ns
Nitrogen fixed per unit pink nodule dry weight (mg g^{-1})			
p=0.041	-	- and +	1118 (547)
	+	- and +	1525 (463)
Nitrogen fixed per nodule (mg nodule^{-1})			
p=0.08	-	-	0.0821
LSD 0.033	-	+	0.0583
	+	-	0.0609
	+	+	0.0780
Nitrogen fixed per pink nodule (mg nodule^{-1})			
p=0.008	-	-	0.1445 **a**
LSD 0.0417	-	+	0.0827 **b**
	+	-	0.1184 **ab**
	+	+	0.1417 **a**

Analysis of uninoculated, N fertilised plants:
no significant differences

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Other nodule number			ns

Burr Medic (Santiago) / Flumetsulam			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**

4.3.5 Burr medic / Imazamox (Table 4.8)

Despite obvious changes to nodulation caused by herbicide application to the plant and also pre-exposure of rhizobia to herbicide prior to inoculating in this experiment, there were no significant effects of either of the treatments on plant dry weight and nitrogen or the amount of nitrogen fixed other than a slight change to biomass partitioning. Pre-exposure of rhizobia to herbicide increased the below ground / shoot dry weight ratio from 0.49 to 0.56 (p=0.042).

Total nodule dry weight and pink nodule dry weight followed a similar trend, whereby unsprayed plants inoculated with unexposed rhizobia had higher nodule dry weight than plants with the other 3 treatment combinations (p=0.002). There was a main effect of plant-applied herbicide which reduced the total number of nodules by 31% (p=0.006), and a trend for the reduction in the number of pink nodules by 24% (p=0.063). Pre-exposure of rhizobia to herbicide reduced the dry weight per pink nodule from 0.25 g to 0.177 g (p=0.024) and the pink nodule dry weight per unit root dry weight from 0.120 to 0.082 g g^{-1} (p=0.002). There were no significant differences in the amount of nitrogen fixed, thus it follows that plant applied herbicide increased the amount of nitrogen fixed on a nodule mass or number basis.

An extra treatment was included in this experiment. Some uninoculated plants were supplied with nitrogen, and the effects of plant applied herbicide assessed. Imazamox consistently reduced root and shoot dry weight and total nitrogen, with no changes to the nitrogen concentration.

Table 4.8 Treatment effects and interactions for the burr medic / imazamox experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Burr medic (Santiago) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			ns
Total plant dry weight (g)			ns
Total plant nitrogen concentration (mg g^{-1}) ns			
Shoot nitrogen (mg)			ns
Shoot dry weight (g)			ns
Shoot nitrogen concentration (mg g^{-1}) ns			
Root + nodule nitrogen (mg)			ns
Root + nodule dry weight (g)			ns
Root + nodule nitrogen concentration (mg g^{-1})			ns
Root : Shoot dry weight ratio			
p=0.042	-	- and +	0.488 (0.092)
	+	- and +	0.562 (0.116)
Root : Shoot nitrogen ratio			ns
Nodule dry weight (mg)			
p=0.002	-	-	5.50 **a**
LSD 1.04	-	+	3.00 **b**
	+	-	2.79 **b**
	+	+	2.97 **b**
Pink nodule dry weight (mg)			
p=0.002	-	-	4.60 **a**
LSD 0.97	-	+	2.44 **b**
	+	-	2.47 **b**
	+	+	2.69 **b**
Nodule number			
p=0.006	I	-	31.3 (11.9)
	I	+	21.7 (4.7)
Pink nodule number			
p=0.063	I	-	17.4 (4.9)

Burr medic (Santiago) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
	I	+	13.3 (5.9)
Other nodule number			ns
Proportion of nodule dry weight that is pink ns			
Dry weight per nodule (mg nodule^{-1})			ns
Dry weight per pink nodule (mg nodule^{-1})			
p=0.024	-	- and +	0.250 (0.109)
	+	- and +	0.177 (0.061)
Nodule dry weight per unit root dry weight (g g^{-1})			ns
Pink nodule dry weight per unit root dry weight (g g^{-1})			
p=0.002	-	- and +	0.1203 (0.052)
	+	- and +	0.0820 (0.041)
Nodule number per unit root biomass (g^{-1})			ns
Pink nodule number per unit root biomass (g^{-1}) ns			
Nitrogen fixed (mg)			ns
Nitrogen fixed per unit nodule mass (mg g^{-1})			
p=0.049	I	-	1791 (810)
	I	+	2277 (644)
p=0.029	-	- and +	1759 (586)
	+	- and +	2308 (840)
Nitrogen fixed per unit pink nodule mass (mg g^{-1})			
p=0.047	I	-	2036 (853)
	I	+	2638 (793)
Nitrogen fixed per nodule (mg nodule^{-1})			
p=0.009	I	-	0.229 (0.074)
	I	+	0.297 (0.064)
Nitrogen fixed per pink nodule (mg nodule^{-1})			
p=0.031	I	-	0.379 (0.081)
	I	+	0.563 (0.287)

Analysis of uninoculated, N fertilised plants			
Total plant nitrogen (mg)			
p=0.016	N	-	4.27 (0.30)
	N	+	3.49 (0.66)
Total plant dry weight (g)			
p=0.022	N	-	0.0909 (0.0136)

Burr medic (Santiago) / Imazamox			
ANOVA	**Inoculation treatment**	**Herbicide treatment**	**Mean (SD)**
	N	+	0.0746 (0.0179)
Shoot nitrogen (mg)			
p=0.013	N	-	1.921 (0.197)
	N	+	1.525 (0.346)
Shoot dry weight (g)			
p=0.07	N	-	0.0431 (0.0066)
	N	+	0.0368 (0.0092)
Root + nodule nitrogen (mg)			
p=0.058	N	-	2.346 (0.259)
	N	+	1.97 (0.368)
Root + nodule biomass (g)			
p=0.008	N	-	0.0478 (0.0076)
	N	+	0.0378 (0.0095)

As explained in the methods section, only the herbicide treatments were analysed in the subterranean and balansa clover experiments.

4.3.6 Subterranean clover / Imazethapyr

There were no significant plant-applied herbicide effects in this experiment.

4.3.7 Subterranean clover / Imazamox

There were no significant plant-applied herbicide effects in this experiment.

4.3.8 Subterranean clover / Flumetsulam (Table 4.9)

Flumetsulam application reduced the shoot dry weight (p=0.022) and the shoot nitrogen (p=0.011) of subterranean clover plants by 20% and 17% respectively. Since root dry weight and nitrogen were not affected there were increases in the root to shoot ratios of biomass (p=0.025) and nitrogen (p=0.012). Plant-applied herbicide reduced the total nodule dry weight (p=0.025) and pink nodule number (p=0.063) by 25% and 28% respectively, and the amount of nitrogen fixed by 16% (p=0.044).

Table 4.9 Herbicide treatment effects for the subterranean clover / flumetsulam experiment. Where significant (p<0.05) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%. For the inoculation treatment, '-' refers to unexposed rhizobia, '+' refers to pre-exposed rhizobia.

Subterranean clover (York) / Flumetsulam			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			
p=0.025	I	-	5.42 (0.92)
	I	+	4.53 (1.11)
Total plant dry weight (g)			

Subterranean clover (York) / Flumetsulam			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
p=0.03	I	-	0.1954 (0.039)
	I	+	0.1675 (0.030)
Total plant nitrogen concentration (mg g^{-1})			ns
Shoot nitrogen (mg)			
p=0.011	I	-	3.81 (0.63)
	I	+	3.03 (0.81)
Shoot dry weight (g)			
p=0.022	I	-	0.1332 (0.028)
	I	+	0.1105 (0.019)
Shoot nitrogen concentration (mg g^{-1}) ns			
Root + nodule nitrogen (mg)			ns
Root + nodule dry weight (g)			ns
Root + nodule nitrogen concentration (mg g^{-1}) ns			
Root : Shoot dry weight ratio			
p=0.025	I	-	0.4379 (0.066)
	I	+	0.4797 (0.034)
Root : Shoot nitrogen ratio			
p=0.014	I	-	0.418 (0.052)
	I	+	0.506 (0.069)
Nodule dry weight (mg)			
p=0.025	I	-	4.85 (1.30)
	I	+	3.65 (0.70)
Nodule number			ns
Pink nodule number			
p=0.063	I	-	23.0 (9.55)
	I	+	16.5 (4.83)
Dry weight per nodule (mg $nodule^{-1}$) ns			
Nodule dry weight per unit root dry weight (g g^{-1}) ns			
Nodule number per unit root dry weight (g^{-1}) ns			
Pink nodule number per unit root dry weight (g^{-1}) ns			
Nitrogen fixed (mg)			
p=0.044	I	-	5.06 (0.95)
	I	+	4.26 (1.14)
Nitrogen fixed per unit nodule dry weight (mg g^{-1}) ns			

Subterranean clover (York) / Flumetsulam			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
Nitrogen fixed per nodule (mg nodule^{-1}) ns			

4.3.9 Balansa clover / Imazamox

Although visual symptoms caused by imazamox application to balansa clover were present, statistically significant differences were only found when plants inoculated with pre-exposed rhizobia (the later inoculation treatment) were analysed on their own. In these plants, herbicide reduced the plant dry weight (p=0.03) by 35% and increased the nitrogen concentration of plants (p=0.003) by 8%. Due to variability, only a trend in total plant nitrogen was evident (p=0.082) with herbicide leading to a reduction of 30%. Trends were also evident in which herbicide reduced the nodule dry weight (p=0.062) and amount of nitrogen fixed (p=0.073).

Table 4.10 Treatment effects and interactions for the balansa clover / imazamox experiment. Where significant (p<0.05) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Balansa clover (Frontier) / Imazamox			
ANOVA of pre-exposed rhizobia			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			
p=0.082	+	-	2.85 (0.84)
	+	+	2.0 (1.12)
Total plant dry weight (g)			
p=0.033	+	-	0.0741 (0.026)
	+	+	0.0485 (0.029)
Total plant nitrogen concentration (mg g^{-1})			
p=0.003	+	-	38.98 (2.75)

Balansa clover (Frontier) / Imazamox			
ANOVA of pre-exposed rhizobia			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
	+	+	42.13 (2.35)
Shoot nitrogen (mg)			
p=0.069	+	-	2.11 (0.66)
	+	+	1.47 (0.80)
Shoot dry weight (g)			
p=0.03	+	-	0.0554 (0.021)
	+	+	0.0359 (0.021)
Shoot nitrogen concentration (mg g^{-1})			
p=0.029	I	-	38.72 (3.21)
	I	+	41.53 (1.62)
Root + nodule nitrogen (mg)			
p=0.013	+	-	0.742 (0.196)
	+	+	0.535 (0.327)
Root + nodule dry weight (g)			
p=0.086	+	-	0.0187 (0.0057)
	+	+	0.0127 (0.0084)
Root + nodule nitrogen concentration (mg g^{-1}) ns			
Root : Shoot dry weight ratio ns			
Root : Shoot nitrogen ratio ns			
Nodule dry weight (mg)			
p=0.062	+	-	1.90 (0.6)
	+	+	1.20 (0.5)
Nodule number ns			
Pink nodule number ns			
Mass per nodule (mg $nodule^{-1}$) ns			
Nodule dry weight per unit root dry weight (g g^{-1}) ns			
Nodule number per unit root dry weight (g^{-1}) ns			
Pink nodule number per unit root dry weight (g^{-1}) ns			
Nitrogen fixed (mg)			
p=0.073	+	-	2.69 (0.85)
	+	+	1.80 (1.1)
Nitrogen fixed per unit nodule dry weight (mg g^{-1}) ns			
Nitrogen fixed per nodule (mg $nodule^{-1}$) ns			

4.3.10 Balansa clover / Flumetsulam (Table 4.11)

Flumetsulam application reduced the dry weight of shoot (p=0.003) and roots plus nodules (p=0.002) of balansa clover by 30% and 27% respectively. Shoot (p=0.006) and below ground (p=0.002) nitrogen declined to a slightly lesser extent, with reductions of 28% and 26% respectively. Flumetsulam reduced the nodule dry weight by 32% (p=0.003), whereas the dry weight per nodule declined by 24% (p=0.018). Flumetsulam reduced the amount of nitrogen fixed by 29% (p=0.003).

Table 4.11 Treatment effects and interactions for the balansa clover / flumetsulam experiment. Where significant ($p<0.05$) treatment means are given, standard deviations are presented in brackets. Where the difference is between more than 2 treatments, letters denote a significant difference using the lsd at 5%.

Balansa clover (Frontier) / Flumetsulam			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
Total plant nitrogen (mg)			
p=0.003	I	-	4.33 (0.88)
	I	+	3.14 (0.84)
Total plant dry weight (g)			
p=0.002	I	-	0.1232 (0.029)
	I	+	0.0867 (0.026)
Total plant nitrogen concentration (mg g^{-1})			ns
Shoot nitrogen (mg)			
p=0.006	I	-	3.31 (0.72)
	I	+	2.39 (0.67)
Shoot dry weight (g)			
p=0.003	I	-	0.0931 (0.023)
	I	+	0.0647 (0.021)
Shoot nitrogen concentration (mg g^{-1})			ns
Root + nodule nitrogen (mg)			
p=0.002	I	-	1.018 (0.20)
	I	+	0.749 (0.19)

Balansa clover (Frontier) / Flumetsulam			
ANOVA	**Rhizobia treatment not tested**	**Herbicide treatment**	**Mean (SD)**
Root + nodule dry weight (g)			
p=0.002	I	-	0.0302 (0.0069)
	I	+	0.0220 (0.0066)
Root + nodule nitrogen concentration (mg g^{-1})			ns
Root : Shoot dry weight ratio			ns
Root : Shoot nitrogen ratio			ns
Nodule dry weight (mg)			
p=0.003	I	-	3.80 (0.9)
	I	+	2.59 (0.7)
Nodule number			ns
Pink nodule number			ns
Dry weight per nodule (mg $nodule^{-1}$)			
p=0.018	I	-	0.155 (0.07)
	I	+	0.118 (0.05)
Nodule dry weight per unit root dry weight (g g^{-1})			ns
Nodule number per unit root dry weight (g^{-1})			ns
Pink nodule number per unit root dry weight (g^{-1})			ns
Nitrogen fixed (mg)			
p=0.003	I	-	4.19 (0.87)
	I	+	2.99 (0.83)
Nitrogen fixed per unit nodule dry weight (mg g^{-1})			ns
Nitrogen fixed per nodule (mg $nodule^{-1}$)			ns

4.4 Summary of results

The experiments completed in this chapter demonstrate the potential for group B herbicides to reduce nitrogen fixation by pasture legumes, and the possible role of rhizobia in determining the severity of the effects. Responses ranged from no significant differences through to a very clear reduction in nodulation and nitrogen fixation. Changes were caused by both plant-applied herbicide and exposure of rhizobia to herbicide prior to inoculation, indicating that the herbicides had some impact on rhizobial function and thereby impacted on the symbiosis. As for the grain legumes in

Chapter 3, there was not a consistent response across the legume by herbicide combinations.

The most obvious way by which herbicides reduced nitrogen fixation was by limiting the growth of the host plant. This was observed when imazamox was applied to lucerne (32%), and where flumetsulam was applied to balansa (29%) and subterranean clover (20%). Imazamox reduced the root dry weight more than the shoot dry weight of lucerne plants, despite the herbicide being applied exclusively to the shoots after the three trifoliate leaf stage. Flumetsulam only reduced the growth of shoots of subterranean clover, but affected both roots and shoots of balansa clover.

Nodulation was generally found to be more susceptible to plant-applied herbicide than the growth of either the shoots or roots. For example, in the lucerne / imazamox experiment the percentage reduction in nodule number and dry weight was greater than that for either shoot or root dry weight. Nodulation of burr medic was negatively affected by both flumetsulam and imazamox, despite only limited changes being detected in the plant dry weight and nitrogen contents. The true extent of differential effects on nodule development compared to other plant organs can't be determined from these experiments due to the timing of herbicide application and variation of growth rates between different plant organs at different times. Plant-applied flumetsulam also reduced the nodule dry weight and nodule number on subterranean and balansa clover and imazamox applied to burr medic reduced nodule number. Therefore it might be expected that the effects of group B herbicides on nodulation of pasture legumes will be widespread.

Pre-exposure of rhizobia to herbicide had mixed effects, in some cases reducing rhizobia fitness and in other cases resulting in a 'protective' effect. As might be expected nodulation was the primary parameter influence by the pre-exposure treatments. In the absence of plant applied herbicide reduced rhizobia fitness following pre-exposure was evident. For example, pre-exposing WSM1115 to flumetsulam lowered the nitrogen concentration of burr medic plants. This may be indicative of a compromised ability of the rhizobia to form symbioses and/or fix nitrogen, which is also supported by the reduction in the pink nodule dry weight per unit root dry weight. Similarly, the total number of nodules on lucerne plants was reduced by pre-exposure of

rhizobia to imazethapyr which suggests that the ability of the rhizobia to infect the host plant was reduced. However the number of pink nodules was not significantly different and the dry weight of roots plus nodules of plants inoculated with pre-exposed rhizobia was also reduced.

In some cases nodulation of plants inoculated with the pre-exposed rhizobia treatment faired worse than their unexposed counterparts where herbicide was applied to the plants as well. For example, the reduction in nodule dry weight per unit root dry weight due to flumetsulam application was only observed in lucerne plants inoculated with pre-exposed rhizobia. For burr medic, imazamox application to plants inoculated with pre-exposed rhizobia reduced the dry weight of pink nodules and total nodules, and a similar situation was seen for lucerne with imazethapyr. Plant applied flumetsulam on burr medic plants inoculated with pre-exposed rhizobia reduced the number of nodules on plants, in contrast to plants inoculated with un-exposed rhizobia, where an increase in nodule number was observed.

The effect of group B herbicides on rhizobia cannot be said to be entirely negative. Exposure of rhizobia to flumetsulam resulted in increases in nitrogen concentration and the amount of nitrogen fixed by lucerne plants. This was linked to more pink nodules on plants inoculated with pre-exposed rhizobia, and a higher amount on nitrogen fixed per unit nodule dry weight. Furthermore, flumetsulam reduced the proportion of total biomass and nitrogen in lucerne shoots, as might be expected due to the topical application of herbicide on the shoots. However this only occurred for plants inoculated with unexposed rhizobia. When inoculated with pre-exposed rhizobia, the partitioning of biomass and nitrogen was the same as that for unsprayed plants inoculated with unexposed rhizobia. This may indicate a protective effect of inoculating with rhizobia that had been previously subjected to herbicide. Pre-exposure of rhizobia to flumetsulam increased the amount of nitrogen fixed per unit pink nodule dry weight on lucerne plants, as did pre-exposure of rhizobia to imazamox for burr medic plants. This indicated a higher efficiency of these nodules. In another example, the nodule size of lucerne plants declined with imazethapyr application to the plant, but only when plants were inoculated with unexposed rhizobia.

There was evidence for improved nitrogen fixation efficiency of nodules with herbicide application, and also exposure of rhizobia to herbicide. This was not directly measured in an assay, however the calculation of the amount of nitrogen fixed on a nodule basis showed that where nodulation was limited by herbicide treatment, it was possible for more nitrogen to be fixed by whatever nodule mass remained (e.g. all lucerne experiments, burr medic / imazamox).

It should be noted that interactions and main effects involving the rhizobia treatment may still be possible for subterranean and balansa clover. Statistical analyses for these experiments were limited to detecting treatment effects of plant applied herbicide.

The screening experiments presented here demonstrated a variety of responses of the pasture legume / rhizobia symbioses to group B herbicides applied to the plant or to rhizobia prior to inoculation. Where more than one herbicide was applied to a single species, different effects were measured. There was little consistency in how the same herbicide affected different pasture legumes, although flumetsulam caused rhizobia pre-exposure effects and interactions in both lucerne and burr medic, and consistent reductions in biomass, nodulation, plant nitrogen and N_2 fixation in balansa clover and subterranean clover.

Chapter 5 Discussion of screening experiments

The experiments outlined in Chapters 3 and 4 add to the paucity of published results that assess the potential of recommended 'in-crop' group B herbicides to inhibit growth and N_2 fixation of grain and pasture legumes. Growth, nodulation and nitrogen fixation were compromised by treatment with group B herbicides as per label recommendations, and by the exposure of rhizobia to herbicide prior to inoculation. Treatment effects are summarised in Table 5.1 which shows the diversity of responses amongst the 17 legume by herbicide combinations.

The experiments conducted on grain and pasture legumes were unique in context with the literature in that they assessed the impact of recommended 'in-crop' applications of group B herbicides and examined more specifically the nodulation and nitrogen fixation in response to plant applied herbicide as well as the exposure of rhizobia to the herbicide. Most published studies on grain legumes have primarily assessed plant symptoms and effects on plant growth and yield (Baerg and Barrett, 1996; Barnes *et al*., 1996; 1991; Wheeler *et al*., 1996; Wilson and Miller, 1991) or were laboratory based mechanistic studies (Gonzalez *et al*., 1996; Royuela *et al*., 2000; Scarponi *et al*., 1996). These studies range from no effects and proclaiming the herbicides to be safe, to showing delayed maturity and stunting. Similarly for pasture legumes, with the exception of Fajri *et al*. (1996), authors have only described internode shortening (Hart *et al*., 1991) or assessed only seed yield (Dickinson *et al*., 1993; Ewers and Phillips,

1993) and professed the recommended 'in crop' group B to be safe. Studies more targeted at nodulation and nitrogen fixation (Anderson, 2001; Eberbach and Douglas, 1989; Eberbach and Douglas, 1991; Martensson, 1992) have only assessed chlorsulfuron which is in the sulfonylurea class of group B herbicides and are therefore not directly comparable to the current study.

In the screening experiments no clear patterns emerged that suggest that certain legumes or certain herbicides are particularly problematic. (Table 5.1) The lack of consistency in effects on a herbicide or legume basis suggests that other factors relating to the actual dose received by the plant may be important. Although most of the treatment effects were detrimental to growth, nodulation and nitrogen fixation, there was some evidence of compensation and protection in response to herbicide application and pre-treatment of rhizobia. Thus a greater understanding of the mechanisms by which group B herbicides can affect nitrogen fixation is required.

Where a reduction in nitrogen fixation was observed in the screening experiments, three types of responses can be envisaged:

1) A clear impact of the herbicide on the plant, with reductions in nitrogen fixation coupled to the inhibition of plant growth;
2) More specific effects on nitrogen fixation that may be due to nodulation and nitrogen fixation being more sensitive to the herbicide than general plant functions;
3) More subtle changes involving the pre-exposure of rhizobia and/or yet to be defined mechanisms.

These scenarios need not be exclusive, and in any situation more than one mechanism may be involved. However the scenarios provide a means to conceptualise the possible mechanisms.

Table 5.1 Summary of treatment effects ($p<0.05$) on nitrogen content, plant biomass and nitrogen concentration of shoots, roots and whole plant samples, as well as nodulation and nitrogen fixation in the grain and pasture legume screening experiments. Note: only the plant applied herbicide treatment was assessed in the balansa clover and subterranean clover experiments.

Significant effects (p<0.05)	Plant Growth and Nitrogen									Nodulation and Nitrogen Fixed								
	Whole Plant			Shoots			Roots			Nodules						N Fixed		
Herbicide (H) Herbicide*Inoculation (H*I) Rhizobia pre-exposure (R) Pre-exposure*Herbicide (R*H)	N	DM	[N]	N	DM	[N]	N	DM	[N]	Nod DM	Pink Nod #	Total Nod #	DM / Nod #	# / Root DM	DM/RootDM	N	N / NodDM	N / Nod#
Chickpea / Imazethapyr	H*I	H*I	H*I	H*I	H*I		H*I	H		H	NA	H	H		H	H	H	H
Chickpea / Flumetsulam	R*H				H		H	H			NA							
Pea / Imazamox	R*H	H	R*H	R*H	H	R*H	R*H		R*H		NA					R*H	R*H	
Pea / Flumetsulam			H*I			H			H*I		NA					H	H	
Pea / Imazethapyr			H								NA	H		H				
Bean / Imazethapyr			R*H				R		R*H		NA							
Vetch / Flumetsulam	H	H	H	H	H	H	H	H			H	H	H	H		H	H	H
Lucerne / Imazamox	H	H	R*H	H	H	R*H	H	H	R*H	H	H	H	R			H	H	
Lucerne / Flumetsulam	R	R	H	R	R	H	R	R			R					R		
Lucerne / Imazethapyr								R				R	R*H					
Burr medic / Flumetsulam			R								R*H	R*H					R	R*H
Burr medic / Imazamox										R*H	H	H			R			
Sub clover / Imazethapyr																		
Sub clover / Imazamox																		
Sub clover / Flumetsulam	H	H		H	H					H	H					H		
Balansa clover / Imazamox		H	H		H	H	H											
Balansa clover / Flumetsulam	H	H	H	H	H	H	H	H		H			H			H		H

N = g nitrogen per plant; DM = g dry matter; [N] = mg N per g DM; # = nodule number; Pink = viable nodules; NA = not assessed

5.1 Effect of AHAS inhibiting herbicides on plant growth and N_2 fixation

It is clear from the inclusion of uninoculated plants in the grain legume screening experiments that group B herbicides can reduce the growth of plants independent of nitrogen fixation. These same 'plant effects' are in turn likely to have implications for nitrogen fixation if the legume is in symbiosis. Where the inhibition of nitrogen fixation and plant growth were tightly coupled, no change in N concentration of the plant tissues would be expected. In the chickpea imazethapyr and pea imazamox experiments the decline in the plant nitrogen and plant dry weight was similar. Similar observations were made by Anderson (2001) under field conditions and Royuela *et al*. (2000) and Gonzalez *et al*. (1996) under controlled conditions. This was also the response of balansa clover and subterranean clover to flumetsulam. In these cases effects on plant growth appear to be the principle way in which group B herbicides reduce nitrogen fixation. Fajri *et al*. (1996) also observed decreases in shoot dry weight of annual medic due to imazethapyr and subterranean clover due to flumetsulam and imazethapyr in the field.

In other experiments an increase in plant nitrogen concentration was observed. In these experiments a decoupling of plant growth and nitrogen fixation can be hypothesized, whereby dry weight gain was reduced more than nitrogen fixation resulting in an increase in the plant nitrogen concentration. Such a response was observed in the balansa clover / imazamox experiment, lucerne / flumetsulam experiment, the vetch / flumetsulam experiment and for lucerne / imazamox plants inoculated with unexposed rhizobia, and would also suggest primary effects of the herbicides to be on plant growth.

The potential of a herbicide to affect growth, nodulation and nitrogen fixation may be related to how the herbicide is applied and whether it is translocated. All three of the herbicides used in the presented experiments are readily translocated between shoots and roots in both the xylem and phloem (Armbrust *et al.*, 2007), hence whether applied post-sowing pre-emergence (PSPE) to roots or pre-emergence to shoots they could potentially affect all plant tissues directly. Where herbicides exist in soils it is either after PSPE application or as residual herbicide from previous applications. In these

cases, symptoms are more likely to be below ground and highly variable due to the heterogeneous nature of soil. The screening experiments showed that the application method did affect the degree to which shoot or root growth was reduced. The PSPE application of imazethapyr to chickpea caused obvious damage to roots and less damage to shoots. In contrast some shoot applied treatments reduced root plus nodule dry weights more than shoot dry weights (flumetsulam/chickpea, imazamox /lucerne). These differences may be attributed to different herbicide translocation and tolerance of each plant species (via detoxification of the herbicide) to each particular herbicide. For example, Dear and Sandral (1999) showed differing tolerance between subterranean clover cultivars to imazethapyr. Similarly in Chapter 4, flumetsulam only reduced the growth of shoots of subterranean clover, but affected both roots and shoots of balansa clover.

5.2 Effect of AHAS inhibiting herbicides on nodulation

Herbicides have the potential to affect nitrogen fixation via mechanisms that are more specific than a general inhibition of plant growth. The most obvious examples of this was where nodulation (nodule numbers or nodule mass) was reduced more by herbicide application than plant growth in general. Similar observations have been reported by Royuela *et al*. (2000), Gonzalez *et al.* (1996) and Drew *et al*. (2007). Where herbicide was applied post emergence it cannot be stated for certain that nodulation was more sensitive than plant growth because the amount of biomass and nodules at the time of herbicide application was not quantified. However where herbicide was applied PSPE, more conclusive evidence exists. For example, in the chickpea / imazethapyr experiment nodule mass in particular was more sensitive to herbicide than root growth. Similarly when imazethapyr was applied either PSPE or post emergent to field pea, there was a reduction in the number of nodules compared to controls. By reducing the number and/or size of nodules, the ability to fix nitrogen may be compromised even if the plant recovers or is not as severely affected as nodulation. Reductions in nitrogen fixation might have become more evident had the experiments progressed further. However, compensation through higher nodule efficiency (discussed below) and the ability of the symbiosis to recover also remains a possibility. Of the more comparable studies associated with group B herbicides, Anderson (2001) found reductions in nodulation and nitrogen fixation in chickpea plants due to imazethapyr, and Gonzalez *et*

al. (1996) found similar results for field pea treated with imazethapyr. Both studies concluded that nodule initiation rather than development was affected. In contrast, the results presented in Chapter 3 in the chickpea / imazethapyr experiment showed that on a root dry weight basis, nodule dry weight was reduced but the number of nodules was not. This indicated that nodule development was affected more than nodule initiation.

Nodule initiation may be interrupted following herbicide application and result in reduced nodule numbers due to a number of factors.

a. While detailed morphological measurements were not undertaken in the screening experiments, in some experiments there were clear changes to root morphology that were likely to have impacts on nodulation. For example PSPE application of imazethapyr caused shortening of roots and arrow tips on chickpea plants similar to the turgid root tips and short, thick roots with few abnormally curved lateral roots described by Royuela *et al*. (2000). Martensson and Nilsson (1989) observed lucerne root hair deformations caused by chlorsulfuron that were expected to inhibit the normal root hair infection process.
b. In the case of post-emergent herbicide applications, the cessation of plant growth caused by inhibition of BCAA synthesis combined with a simultaneous perturbation of free amino acids, may result in a down regulation of nitrogen fixation due to less plant demand for N (Hartwig and Trommler, 2001). Subsequently nodulation following herbicide application may be reduced or cease.
c. Reduced growth or crop yellowing may reduce photosynthesis and the subsequent supply of carbohydrates to the roots and nodules. In Chapter 3, flumetsulam caused slight yellowing of leaves on pea and despite a lack of measurable growth inhibition, reduced the amount of nitrogen fixed by 31%. This is in agreement with Drew *et al*. (2007) where the degree of yellowing of leaves on pea plants sprayed with various herbicides (including group B) was correlated to a decrease in nodulation in field experiments. In another case Sprout *et al.* (1992) found the photosystem-II inhibiting herbicide metribuzin stopped the formation of nodules on lentil for 10 days before it rapidly resumed (presumably following herbicide metabolism).

Conversely to less nodules being formed, existing nodules may senesce or shed which would also result in a lower nodule number and dry weight. A stress response by the legume host (e.g. water deficit) has been shown to induce nodule senescence and shedding (Matamoros *et al*., 1999; Venkateswarlu *et al*., 1989). While this cannot be directly concluded from the results in Chapters 3 and 4, a dramatic reduction in the number of pink nodules was evident in the vetch / flumetsulam experiment and is consistent with the senescence or shedding of existing nodules. It is entirely possible that herbicide application resulting in reduced growth may indeed invoke a stress response in the host plant.

In some experiments there was evidence for improved nitrogen fixation efficiency of nodules with herbicide application, and also exposure of rhizobia to herbicide (discussed below). This was not directly measured in an assay, however the calculation of the amount of nitrogen fixed on a nodule basis showed that where nodulation was reduced by herbicide treatment, it was possible for more nitrogen to be fixed by whatever nodule mass remained (e.g. all lucerne experiments, burr medic / imazamox). Explanations for this include: more carbohydrate available to the existing nodules; nodules that were once active were shed and hence not counted or weighed; and nodules formed on control plants after the time of herbicide application were not as efficient. An assay to determine the specific activity of nodules is required to clarify this result.

5.3 Effect of AHAS inhibiting herbicides on rhizobia

The *in vitro* experiments in this work and in the literature consistently show a lack of rhizobial growth inhibition caused by group B herbicides at concentrations relevant to field application rates. It is therefore often assumed that any impact of group B herbicides on nitrogen fixation occurs via the plant. However, it has been shown that exposure of rhizobia to herbicide prior to inoculating can reduce the nodulation of legumes (Anderson *et al*., 2004). This pointed to a role of rhizobia in the inhibition of nitrogen fixation due to group B herbicides, which had not been reported elsewhere. Assuming that no herbicide was carried over in the rhizobial inoculum as previously shown (Anderson *et al*., 2004), the interactions between plant-applied herbicide and pre-exposure of rhizobia showed that the effects of herbicides on nitrogen fixation are

not mechanistically restricted to effects on the plant. Possible explanations include the disruption chemical signalling between the microbes or between the microbes and the host plant, in turn affecting nodulation. Alternatively other biochemical pathways essential for nodule development and N_2 fixation may be compromised. The treatment effects imposed by the exposure of rhizobia to herbicide prior to inoculation suggest such mechanisms may be involved, although the overall contribution to reductions in nitrogen fixation may be minor.

Overall the screening experiments presented here did not reveal a detrimental rhizobia mediated effect to the extent that Anderson *et al.* (2004) described for chlorsulfuron and chickpea. Nevertheless, reduced fitness of rhizobia following pre-exposure to herbicide was apparent in both the absence and presence of plant applied herbicide. This took place in the form of reduce plant N concentrations (e.g. faba bean/imazethapyr, flumetsulam / burr medic), reduced nodule dry weight (e.g. flumetsulam / burr medic, imazethapyr / lucerne) and reduced nodule numbers (imazethapyr / lucerne, flumetsulam / burr medic). Observed differences may in part reflect the sensitivity of different rhizobia strains to the various herbicides.

In contrast to the negative effects described above, pre-exposure of some rhizobial strains by herbicide combinations resulted in a 'protective' effect on the rhizobia, particularly in the presence of plant applied herbicide. In some cases, pre-exposure of rhizobia to herbicide prevented a decline in plant nitrogen and nodulation that occurred when herbicide was applied to plants inoculated with unexposed rhizobia (field pea / imazamox, flumetsulam /lucerne) and in other cases increased N_2 fixation efficiency of nodules (flumetsulam / lucerne, imazamox / burr medic). Indeed, Royuela *et al*. (1998) proposed that rhizobia provide some protection to the macrosymbiont against the inhibition of branch chain amino acid synthesis caused by group B herbicides. It has been hypothesized that a higher specific activity of AHAS in bacteroids enables nodulated plants to better tolerate group B herbicides (Royuela *et al*., 1998). Some results in the screening work might be explained if pre-exposure of rhizobia to herbicide exerted selective pressure on the rhizobial AHAS enzyme.

5.4 Conclusions from screening experiments

The experiments presented here clearly show that group B herbicides can inhibit nitrogen fixation by both grain and pasture legumes. Reductions in nodulation and nitrogen fixation of up to 60% were observed with herbicide application at label rates. Where large reductions in nitrogen fixation occurred, they were generally accompanied by obvious symptoms on the plant and/or stunted growth. The results from this work concur with the literature in that they point to the intolerance of the host legume to herbicide as the primary mechanism of reductions in nitrogen fixation. The fact that herbicide impacts were observed on uninoculated plants also indicates that plant growth and function was an important susceptibility in the symbiosis.

As widely shown elsewhere there was no measurable effect of herbicide on the growth of rhizobia in broth cultures at likely field concentrations. The pre-treatment of rhizobia with herbicide prior to inoculation only had minor effects. This was in contrast to the large reductions in nitrogen fixation observed by Anderson *et al*. (2004) with the sulfonylurea herbicide chlorsulfuron.

The variety of responses can be attributed to the specific intolerance of each legume species and rhizobial strain to each herbicide, and the complexity inherent in the nitrogen fixation process. It also indicates the possibility of there being multiple mechanisms by which group B herbicides can affect nitrogen fixation. The variability in the results is consistent with field based variety trials (L. McMurray, personal communication) which show different levels of tolerance to herbicide even amongst cultivars of the same species of legume.

In a field situation, a myriad of edaphic and climatic conditions are likely to influence the net effect of a herbicide on symbiotic nitrogen fixation. Although it is difficult to extrapolate the findings from these experiments to the field, the results nonetheless indicate that some legume by herbicide combinations are likely to alter N fixation less, and may therefore present an opportunity to manage the problem. More detailed assessment of nodulation and biomass production and nitrogen content in herbicide field trials may be beneficial in making more sensible herbicide recommendations to growers. The work described in this chapter is confirmation that there is potential for group B herbicides to disrupt nitrogen fixation. This should be considered by growers

for the management of soil fertility but is difficult to quantitatively assess. Where group B herbicides are used, growers should be aware that provision of N to subsequent crops may be affected.

5.5 Methodological considerations

Various methods exist for assessing nitrogen fixation (Peoples *et al*., 1989). The acetylene reduction assay has been a popular measure of nitrogenase activity, but its use for extrapolating to an amount of nitrogen fixed has come under scrutiny (Minchin *et al*., 1994; Vessey, 1994). ^{15}N methods are particularly useful for field experiments where conditions are conducive. However it can be expensive to use and requires access to specialised equipment. In the screening experiments a simple method of accounting for the amount of non-fixed nitrogen in the system was used. By growing plants in sand and using a nitrogen free nutrient solution, it was possible to account for the amount of seed nitrogen and whatever extraneous nitrogen might be available to plants in the system by measuring the amount of nitrogen in uninoculated plants. To account for the reduced ability of roots to explore the growth media and take up what little nitrogen may occur in the growth medium where herbicide was applied, herbicide was applied to uninoculated plants and used to calculate the amount of nitrogen fixed by inoculated plants with herbicide treatment. Unsprayed uninoculated plants were used to calculate the amount of nitrogen fixed by inoculated unsprayed plants. This was calculated for each block. This method does not account for nitrogen that may be lost from the plant, in exudates and sloughed off root cap and necrotic cells or unrecovered leaf drop, for example. By using Kjeldahl digests and an auto analyser to measure plant nitrogen, the experimental system provided an inexpensive and robust method for calculating the amount of nitrogen fixed by the various legumes in glasshouse experiments. However growth of legumes in a nitrogen free system makes it difficult to ascertain whether herbicide reduced plant growth and hence limited the amount of nitrogen fixed, or nitrogen fixation was impeded first and the N limitation resulted in reduced growth. Some clues are given by the nitrogen concentration of shoots and roots. Future work might be informed by the measurement of the carbon content to determine the elemental stoichiometry and whether nitrogen is limiting.

The inclusion of uninoculated plants in the screening experiments served a number of purposes. The presence of nodules on uninoculated plants was an effective check for contamination by rhizobia. Using this check it was apparent that a number of experiments were contaminated and all such experiments were repeated. A small number of small white nodules were found on uninoculated plants in the chickpea flumetsulam and vetch experiments, however based on their appearance they would not have contributed to N_2 fixation. The second role for uninoculated plants was in providing the basis from which the amount of nitrogen fixed could be calculated, as explained in the methods section. Thirdly, where growth of uninoculated plants was sufficient, the detection of symptoms or growth limitation due to herbicides points to a definite plant effect of the applied herbicide, independent of any influence on nitrogen fixation. Whilst the detection of treatment effects on uninoculated plants does not rule out any rhizobia mediated effects, it does highlight plant intolerance as a mechanism for reduced nitrogen fixation. This third feature was useful in the grain legume experiments, where the seed nitrogen was sufficient to support some growth of uninoculated plants.

Many factors influence the affect of herbicides on nitrogen fixation. Selectivity of the herbicides can be determined not only by resistance which would be conferred by a mutated AHAS enzyme, but also by the ability of a plant to exclude the herbicide or metabolise the active ingredient to an inactive metabolite. Thus the selectivity varies amongst cultivars.

A number of environmental parameters are known to affect the efficacy of herbicides. The complex interactions that can occur are outlined in Green and Strek (2001) and include humidity, light (including ultraviolet light), temperature, soil moisture and leaf surface area and angle. Each of these factors will cause variation between experiments, and indeed the extrapolation of results from this screening work to the field is problematic. Herbicide can also be taken up by the roots and a number soil factors would influence the dose that plants and rhizobia / bacteroids could be exposed to via this route. Important soil properties include pH, soil water content, clay content, organic matter content, soil temperature and microbial activity.

Various effects of temperature on efficacy of group B herbicides have been reported, and are likely to depend on the herbicide and the species, and also the experimental methodology used (Madafiglio *et al*., 2000).

Where possible, herbicide was applied at a time to allow sufficient light for metabolic activity and uptake of the herbicide by the plant. The time of herbicide application in this work did vary between experiments and therefore may contribute to inter-experimental variation. The actual dose to each plant will also vary depending on the interception of herbicide (leaf angle, surface area, height etc) and the amount of herbicide that is washed into the sand by watering. However the uniformity of plants prior to post-emergent treatment was ensured by selective thinning within experimental blocks so that plant size would be consistent.

The time of day of herbicide application to the plant was particularly important for pasture species, where the leaf angle becomes more acute in low light. This was particularly important on clovers and medic plants. The changes in leaf angle alter the surface area exposed to herbicide droplets, which could reduce the effective dose. This is likely to occur in the morning and evening, or on overcast days. Climatic conditions are also likely to alter the plant's metabolic activity and physiology, which will affect the uptake of herbicide as well as its translocation and metabolic inactivation. Another consideration in the pasture legume experiments was that the lids on the jars prevented any herbicide entering the sand and contacting the roots; a process that may occur in a field situation. Thus the effective dose in these experiments was likely to be lower than what would occur under field applications.

There was little evidence to support the argument that the pre-exposure of rhizobia treatment can cause plant effects via carryover of herbicide in the inoculum. In the burr medic / imazamox experiment, pre-exposure of rhizobia to herbicide actually shifted biomass partitioning towards the roots, which runs contrary to the notion that carryover of herbicide in the inoculum can injure the plant. Anderson *et al.* (2004) showed that carryover of herbicide with the inoculum was negligible, and unlikely to cause injury to the plant. A possible effect not yet considered is that low doses of herbicide may have a 'safener' effect whereby defence responses and priming of herbicide degradation

pathways are initiated and subsequent application of herbicide results in less severe effects.

Liquid cultures differ from soils in a number of ways. Thus the relevance to field situations of the assays conducted *in vitro* in this work can be questioned. The first difficulty lies in replicating the availability of herbicide that may occur in soil. Soil is a matrix of minerals and organic matter and contains fluctuating amounts of air and water. A theoretical example of how variable concentrations of herbicide in the soil can be is explained. If a herbicide accumulates in the top 1 cm of soil, on a soil volume basis it may be 10 times more concentrated than if it is distributed through 10 cm. If it accumulates in 1 mm, it's 100 times more concentrated. If the herbicide remains in solution, a decrease in soil water content will concentrate the herbicide possibly by an order of magnitude. Herbicides can sorb to the soil or organic matter and can therefore persist. Herbicides can also move in the soil, and may accumulate or disperse. Herbicides are also subjected to chemical hydrolysis and microbial decomposition. Soil is heterogeneous so unlike liquid cultures, niches can develop and a spectrum of herbicide availability to degrading organisms, saprophytic rhizobia and legumes can occur. This high spatial heterogeneity means that niches of low or high herbicide availability exist.

Liquid cultures are also designed to maximise the growth of a single strain. The availability of energy and nutrients in a liquid culture might allow microorganisms to withstand chemicals that would otherwise be toxic. Thiamine and pantothenate were discovered to be related to the branch chain amino acid synthesis pathway, and may perhaps contribute to the lack of growth inhibition of rhizobia by the group B herbicides since it was supplied in the media. However it has been demonstrated that supplementation of BCAA auxotrophs with pantothenate did not rescue nodulation (Aguilar and Grasso, 1991). In the soil, certain organisms within a diverse community may degrade the herbicide and thus protect or allow the maintenance of the activity of susceptible organisms.

The effects of herbicides in the screening work were measured on plants at a single sampling time. To better understand the effects of group B herbicides on nodulation in general, a time series study that measures nodule number and mass, nitrogenase activity and compares different timings of herbicide application is warranted. This will help determine whether nodule initiation or development is inhibited by herbicide.

5.6 Exploring mechanisms

The screening experiments established that group B herbicides can inhibit nitrogen fixation. Although the principle mechanism appears to be related to the susceptibility of the plant to the herbicide there is evidence to suggest that other aspects of the symbiosis may be involved. The following chapters describe experiments designed to understand the mechanisms by which group B herbicides inhibit nitrogen fixation.

In Chapter 6 a herbicide tolerant cultivar of *Medicago littoralis* was compared with a near isogenic herbicide susceptible variety. This was expected to highlight non-plant related effects of herbicide on the symbiosis, and also assess nitrogen fixation by this new cultivar that may become a useful agronomic response to the issue of group B herbicide inhibition of nitrogen fixation.

In Chapter 7, the effects of two group B herbicides on the morphology and proteome of medicago roots were explored using proteomics analysis and observations.

Chapter 6 Herbicide tolerant *Medicago littoralis*

6.1 Introduction

A new strand medic (*Medicago littoralis)* cultivar called FEH-1 (Heap, 2000) was derived from the variety Herald by mutagenesis. It is now being commercialised as the variety 'Angel'. It was developed by mutating Herald seed with ethyl methanesulfonate and selecting plant genotypes showing field tolerance to chlorsulfuron. FEH-1 displays tolerance to various group B herbicides. A single mutation in the AHAS gene was identified as the mechanism of tolerance (Oldach *et al*., 2008). FEH-1 was seen as a potential tool for deciphering the mechanisms causing group B herbicide induced reductions in nitrogen fixation. It was envisaged that by using FEH-1 to remove any plant intolerance component, it would be possible to identify and quantify any non-plant related components of the symbiosis that are affected by herbicide and could therefore be implicated in the reductions in N_2 fixation.

FEH-1 was developed to circumvent the problem of soil residues of group B herbicides, particularly chlorsulfuron and triasulfuron, that severely restrict the growth of medicago plants in the year following herbicide application to cereals. *In vitro* assays of the target enzyme acetohydroxyacid synthase from FEH-1 showed increased resistance to chlorsulfuron, flumetsulam, imazethapyr, metsulfuron methyl, triasulfuron and sulfometuron methyl (Heap, 2000). Pot experiments measuring shoot dry weight also showed that FEH-1 had tolerance to group B herbicides compared with Herald, especially to chlorsulfuron, triasulfuron and sulfometuron methyl (Heap, 2000). Field experiments with the herbicide triasulfuron have similarly shown FEH-1 to have greatly improved shoot biomass, seed yield and plant regeneration at up to 26 g triasulfuron ha^{-1}, compared to Herald (Howie and Bell, 2005).

In addition to the inhibition of growth, residual group B herbicides have caused decreased nodulation in medicago plants with the potential to reduce the amount of N_2 fixed. Concentrations equivalent to 0.02 g ha^{-1} of chlorsulfuron reduced the number of nodules on medicago plants compared with zero herbicide controls (Rovira *et al*., 1993). In this case the reduction in nodule number was directly related to the reduction in root length. That the exposure of rhizobia to herbicide resulted in significant treatment effects in the screening experiments suggests that even if plant growth in the presence of herbicide is unaffected in the tolerant FEH-1, N_2 fixation may still be reduced via an impact on the rhizobia or the symbiosis. All testing of FEH-1 to date has been in soil

containing an adequate supply of N (Heap, 2000; Howie *et al*., 2002), and no specific assessment of the ability of the FEH-1 / rhizobia symbiosis to fix nitrogen has been undertaken.

The objectives of the work presented in this chapter were:

1) To use FEH-1 as a tool to separate the plant mediated effects of herbicide from other impacts of herbicide on N_2 fixation.
2) To assess the N_2 fixation capability of herbicide tolerant medicago cultivar FEH-1 compared with near isogenic Herald in the absence and presence of a group B herbicide.

6.2 Methods

6.2.1 Rhizobia experiment – impacts of chlorsulfuron on *in vitro* growth of rhizobia

An experiment was conducted to identify direct effects of chlorsulfuron (CS) on the growth and survival of rhizobia *in vitro*. Cultures of the commercial inoculum *Sinorhizobium meliloti* strain RRI128 were grown in defined media as used in Chapter 3 and 4, but without thiamine and pantothenate in the vitamin supplement. These were omitted because their synthesis is closely related to the branched chain amino acids (Ottenhof *et al*., 2004).

Triplicate flasks containing no herbicide (control), a 'standard' concentration of CS herbicide that approximated levels applied to soil (herb), or a 'high rate' of herbicide (hi herb) were prepared. Filter sterilised (0.2 µm) technical grade chlorsulfuron (CS) was added at 1.5×10^{-8} g ml^{-1} of rhizobial culture (herb) to approximate the recommended Glean ® (Dupont) application rate of 20 g ha^{-1} to a depth of 10 cm. At the higher rate (hi herb), this herbicide was added at 1.5×10^{-6} g ml^{-1} of culture. All flasks were inoculated with 1 ml of a starter culture of RRI128.

The optical density of the cultures was measured at 500 *n*m using a Beckman DU 640 spectrophotometer and disposable cuvettes. This was done after 30 and 42 hours of incubation at 25°C in a shaking incubator. In addition, serial dilutions of cultures were

made and colony forming units counted on drop plates after 48 hours of incubation using the method of Miles and Misra (1938).

6.2.2 Plant experiment – impacts of chlorsulfuron on growth, nodulation and nitrogen fixation of tolerant and intolerant medic

A factorial experiment comprising two plant types, five inoculation treatments and two rates of herbicide application was conducted. Plants were grown in a mix of 50:50 coarse washed sand and vermiculite, in 130 mm diameter pots, which had previously been sterilised by autoclaving.

Two varieties of *Medicago littoralis* were grown. Seed of Herald and FEH-1 (a herbicide tolerant derivative of Herald) were surface sterilised in 4% hypochlorite, rinsed 10 times in sterile water and then germinated overnight at 27°C (as described previously in Chapter 4). Germinated seedlings were transferred to pots containing sterile potting mix, 5 to 6 seedlings per pot. Plastic beads were aseptically applied to the pot surface to reduce evaporation and cross-contamination during watering. Pots were watered with 300 ml ¼ strength McKnight's solution (McKnight, 1949) containing 4 mg of ammonium nitrate as starter N. It became apparent as the experiment progressed that additional N was present as a contaminant in the potting mix. This provided a limited supply of nitrogen that was consistent across the experiment and did not unduly alter the experimental outcomes. Pots were transferred to the greenhouse which was maintained at 25°C during the day and 15°C at night. After three days of growth, seedlings were thinned to two plants per pot to achieve uniform seedling size within each block.

There were 5 'inoculation' treatments (Table 6.1). Two of these treatments were uninoculated, one of which was given a non-limiting supply of ammonium nitrate (denoted O+N) and the other was not given any additional nitrogen (O–N). There were two inoculation treatments in which *Sinorhizobium meliloti* strain RRI128 was centrifuged and washed as described below. One of these had herbicide in the flask during the incubation as described above (herb) in the rhizobia experiment, while the other had no herbicide (washed). To wash the cells, cultures were centrifuged in a

Sorvall RC 5C Plus centrifuge at 10000 rpm in a SS34 rotor, the supernatant discarded, and the pellet resuspended in phosphate buffered saline (PBS). This was repeated 3 times. An additional rhizobia treatment, in which plants were inoculated with untreated culture (no exposure to herbicide or washing), was included as an extra control (RRI128).

Table 6.1 Inoculation treatments

Inoculation treatment	Rhizobia	Herbicide in culture	Washing procedure	PBS suspension	Nitrogen supplied
Uninoculated (O-N)	n	n	n	**y**	n
Uninoculated (O+N)	n	n	n	**y**	**y**
RRI128 (RRI128)	**y**	n	n	n	n
RRI128 (washed)	**y**	n	**y**	**y**	n
RRI128 (herb)	**y**	**y**	**y**	**y**	n

The optical density of all cultures was measured and used to adjust the volumes that the cultures were resuspended in to standardise the number of cells applied to plants in each rhizobia treatment. Inoculum was added to the pots four days after the seedlings were planted. Approximately 10^9 colony forming units were pipetted on to the potting mix surface immediately surrounding each seedling. For the 'herbicide' and 'washed' treatments, cells were resuspended in phosphate buffered saline (PBS). For uninoculated plants (O-N and O+N) an equivalent volume of PBS was applied. For the 'RRI128' treatment, inoculum was applied as non-manipulated culture in the growth medium (i.e. no washing)

Counts of resuspended cells used for inoculating plants showed that each treatment received slightly different numbers of cells. Plants inoculated with RRI128 received 1.99×10^9 colony forming units, washed rhizobia 1.38×10^9 and herbicide treated rhizobia 1.63×10^9 colony forming units per seedling. While these were significantly different from each other ($p<0.001$), they were vastly in excess of that required for unrestricted nodulation and the difference would not have contributed to inoculation treatment effects.

Pots were randomised in the glasshouse, with additional non-experimental pots on the ends of each block to eliminate edge effects.

There were two plant-applied herbicide treatments, including a non herbicide control. Chlorsulfuron was applied at the 3-5 trifoliate leaf stage at the minimum rate found to cause depressed growth in Herald but not in FEH-1 by Heap (2000). This rate was 0.75 g ha^{-1} of CS, which was 5% of the recommended application rate of Glean ® to cereals. Herbicide was sprayed onto 17 day old seedlings using a spray cabinet equipped with an air compressor and agricultural nozzles to simulate field spray equipment. Herbicide was not applied to control plants.

Commencing ten days after herbicide application, plants were harvested in blocks over four days to allow sufficient time to conduct acetylene reduction assays (ARA) immediately following the washing of roots. After washing sand from the roots, plants were wrapped in moist paper towel and taken immediately to the laboratory with the ARA equipment. The shoots of two plants in each pot were excised and the roots placed in glass jars (Figure 6.1). Air tight lids were screwed on and 25 ml of instrument grade acetylene (C_2H_2) injected through a septum in the lids. After exactly 60 minutes of incubation at 23°C (room temperature), samples of the head space gases were taken in syringes and transferred to vacutainers. 1 ml samples were then injected manually into a Varian CP 3380 gas chromatograph fitted with an F.I.D. and 2.5 m stainless steel column packed with porapak N (80/100). The column temperature was 110°C, detector temperature 120°C and the carrier gas flow rate was 30 ml of N_2 per minute. Industrial grade ethylene (C_2H_4) was used as an external standard and C_2H_4 concentrations in the incubation chamber were calculated using peak area and appropriate correction factors.

Figure 6.1 Incubation jars for acetylene reduction assays

Root systems were stored in moistened plastic bags at 4°C until nodules were removed and counted. Nodules were divided into effective (pink) and ineffective (non-pink) classes based on their appearance, and the number of nodules in each class was counted. The dry weight of shoots, roots, pink and other nodules was measured after drying in an oven at 50°C for at least 48 hours. The N contents of shoots and combined root plus nodule samples were determined by Kjeldahl digest (as described in Chapter 3). The nitrogen contents of FEH-1 and Herald seed samples used in the experiment were also determined by Kjeldahl digest. The amount on nitrogen fixed was calculated using the N difference method (Peoples *et al*., 2002).

N_2 fixed = (N content of fixing plant) – (N content of non-fixing control)

The amount of nitrogen fixed by each inoculated plant was calculated by subtracting the amount of N in the O-N plant with the same herbicide treatment in the same block.

Experimental design and statistical analyses

The experiment was arranged in five blocks in the greenhouse with a representative from each of the 20 treatment combinations (2 variety * 2 herbicide* 5 inoculation treatments) randomly assigned to each block. Data from the two plants in each pot were

averaged prior to analysis. Biomass and N data were analysed by analysis of variance (ANOVA) using a 2x2x5 (variety x herbicide x inoculation) factorial treatment structure for experimental units arranged in a randomised complete block design. Plots of residuals were checked for homogeneity of variance.

Uninoculated plants were checked for nodulation and acetylene reduction activity to ensure zero values. These were subsequently removed from the analysis of nodulation and ARA data, and an ANOVA using a 2x2x3 (variety x herbicide x rhizobia treatment) structure for experimental units arranged in a randomised complete block design. Plots of residuals were checked for homogeneity of variance.

Main effects are only presented where the treatment in question was not involved in an interaction with other treatments.

Regression analysis of inoculated plants was used to further understand relationships between plant dry weight, nitrogen, nodulation and nitrogenase activity. F tests on variance ratios were used to determine the most appropriate model for each relationship.

All statistical analyses were completed using Genstat 5 (Release 4.1 1997 VSN International Ltd: Hemel Hempstead, UK).

6.3 Results

6.3.1 *In vitro* exposure of rhizobia to chlorsulfuron

Optical density readings of cultures after 30 and 42 hours of incubation showed a small but significant difference in the optical density of cultures averaged over the two time points (p=0.005). Cultures with herbicide (n = 3) had lower optical density (0.680 for 15 *n*g ml^{-1} chlorsulfuron and 0.698 for 1.5 µg ml^{-1} chlorsulfuron) than cultures without herbicide (0.721) (n=6). However there were no significant differences between treatments for drop plate counts of 42-hour cultures. There were also no significant differences in the ratio of viable cells to optical density of the 42 hour cultures.

Table 6.2 Optical density (A500) and counts (cfu/ml) of RRI128 cultures. A500 readings are an average of 30 and 42 hours, and treatments with different letters are significantly different. The cfu/ml and ratio of cfu to optical density are for cultures sampled at 42 hours only.

Treatment	Chlorsulfuron	Reps	A500	cfu/ml	Ratio
Hi herb	15 ng ml^{-1}	3	0.698 (bc)	2.64E+09	2.22E+09
Herb	1.5 µg ml^{-1}	3	0.680 (c)	2.10E+09	1.78E+09
Wash	0	3	0.712 (ab)	2.94E+09	2.43E+09
Control	0	3	0.730 (a)	2.50E+09	1.79E+09
P value			0.005	0.882	0.886
LSD 5%			0.025	1.35E+09	1.04E+09

6.3.2 Effect of chlorsulfuron on Herald and FEH-1

Results of the experiment assessing the effect of CS on growth, nodulation and N_2 fixation of Herald and FEH-1 are presented in three separate sections. First the biomass and N data for the whole plant, shoots and root plus nodules are presented, along with the partitioning of biomass and N between shoots and roots. The effects of the applied treatments on nodulation will then be presented using nodule counts and dry weights of nodules assessed as pink or non-pink. The nodule data presented on a root dry weight basis will then be examined. Finally, N_2 fixation as assessed by the acetylene reduction assay and the N difference method will be presented.

6.3.3 Plant growth and nitrogen status

The plant variety, inoculation treatment and herbicide treatments that significantly affected plant dry weight and nitrogen status ($p<0.05$) are presented in Table 6.3. Interactions between the herbicide treatment and either the variety or inoculation treatment account for the majority of significant effects observed. Where interactions occurred, the main effects are not presented. Significant differences ($p<0.05$) and trends ($0.05<p<1$) are discussed in more detail.

Table 6.3 Significant main effects and interactions between variety, inoculation and herbicide treatments on plant growth and nitrogen status ($p<0.05$) as determined by analysis of variance. Where interactions are significant, the individual main effects are not presented. *Trends of interest are shown in parentheses.

Variable	Variety (V)	Inoculation (I)	Herbicide (H)	Variety*Herbicide (V*H)	Inoculation*Herbicide (I*H)	Variety*Inoculation (V*I)	Variety*Herbicide*Inoculation (V*H*I)
Total plant (V*I*H)							
Dry weight				<0.001	<0.001		
Nitrogen				<0.001	<0.001		
Nitrogen concentration					0.005		*(0.064)
Shoots (V*I*H)							
Dry weight				<0.001	<0.001		
Nitrogen				<0.001	<0.001		
Nitrogen concentration							0.023
Root plus nodule (V*I*H)							
Dry weight				0.004	0.012		
Nitrogen	<0.001				<0.001		
Nitrogen concentration				0.002	0.019		
Partitioning (V*I*H)							
Root : Shoot biomass		<0.001		<0.001			
Root : Shoot nitrogen				<0.001	0.012		

6.3.3.1 Plant Biomass

In the absence of plant-applied herbicide, shoot and root plus nodule dry weight was greater in Herald plants than in FEH-1 plants. Chlorsulfuron reduced the root and shoot dry weight of Herald plants more so than FEH-1 plants. FEH-1 had higher shoot dry weight than Herald when herbicide was applied, however the root plus nodule dry weight of Herald and FEH-1 plants to which herbicide was applied was not significantly different from each other (Figure 6.2 A).

Inoculation by herbicide interactions for shoot, root plus nodules, and total dry weight (Figure 6.2 B) were caused primarily by the uninoculated plus nitrogen treatment (O+N), in which the total dry weight of unsprayed plants was substantially higher than all the other inoculation by herbicide combinations. The addition of nitrogen to uninoculated plants increased shoot dry weight, but decreased root dry weight if herbicide was applied.

Amongst the inoculated plants to which herbicide was applied, there were no significant differences in shoot or root plus nodule dry weight due to rhizobia treatment. However the rhizobia treatment did significantly affect shoot dry weight of unsprayed plants, with plants inoculated with washed rhizobia accumulating more shoot biomass than plants inoculated with unmodified rhizobia culture.

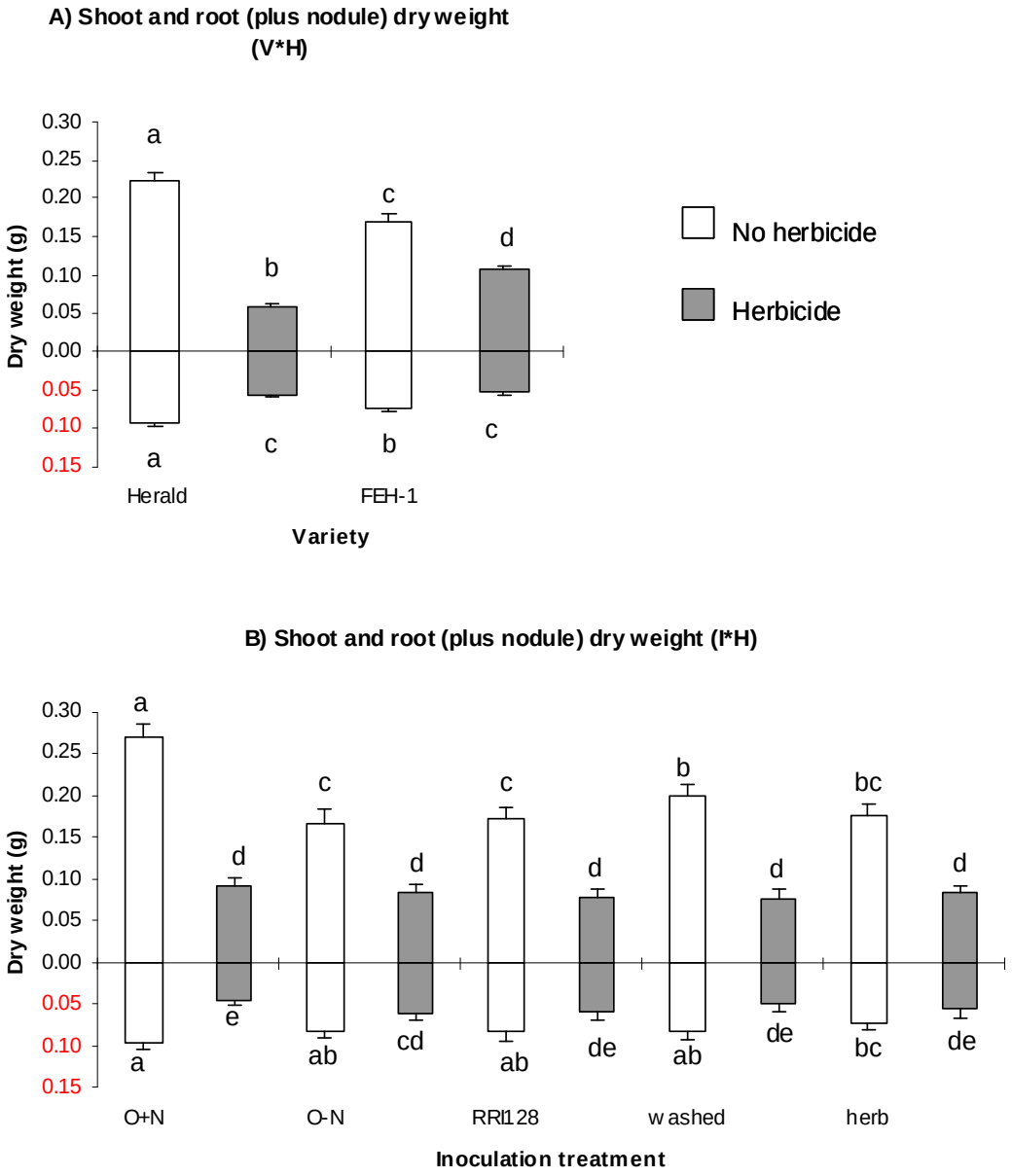

Figure 6.2 Plant dry weight. Shoot dry weight above the x-axis, roots plus nodules below the x-axis. (A) Variety by herbicide interactions ($p<0.001$, lsd5% = 0.015 g for shoots and 0.008 g for roots plus nodules). (B) Inoculation by herbicide interactions ($p<0.001$, lsd5% = 0.024 g for shoots and 0.013 g for roots plus nodules). Error bars indicate standard error of mean.

The partitioning of biomass and N was expressed by dividing the root (plus nodule) dry weight by the total plant dry weight to give the proportion of total dry matter or N in roots. Therefore the higher the value, the greater the amount of biomass or N in below ground organs. Chlorsulfuron application to Herald resulted in a greater portion of

biomass in roots compared with unsprayed Herald and both FEH-1 treatments, all of which had similar biomass partitioning of around 0.3 (Figure 6.3A).

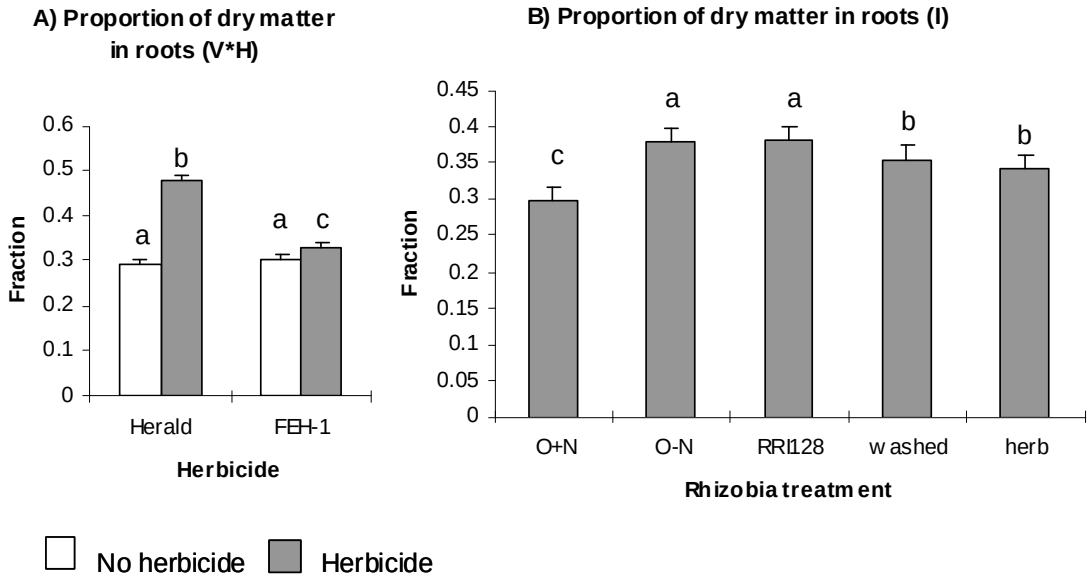

Figure 6.3 Partitioning of biomass between shoots and recovered roots plus nodules. (A) Variety by Herbicide interaction ($p<0.001$, lsd5% = 0.020). (B) Main effect of inoculation treatment ($p<0.001$, lsd5% = 0.023). Bars indicate standard error of mean.

The inoculation treatment also affected the partitioning of biomass between roots and shoots. O+N plants had a lower proportion of the total plant dry matter in roots than the other treatments. In contrast, O-N plants, along with plants inoculated with RRI128 culture had high proportions of dry matter in below ground organs. Both rhizobia treatments that underwent the washing procedure (washed and herb) had similar dry matter partitioning, intermediate to the other inoculation treatments (Figure 6.3B).

6.3.3.2 Nitrogen

Shoot and total plant N followed similar trends to the biomass data (Figure 6.4A), however FEH-1 plants had significantly lower nitrogen in roots plus nodules across the herbicide treatments. Herald had greater shoot N than FEH-1 in the absence of plant-applied herbicide, but less when herbicide was applied.

The addition of nitrogen to unsprayed, uninoculated plants resulted in much greater total plant N compared with the other inoculation treatments (Figure 6.4B). This resulted in

herbicide by inoculation interactions for total, shoot and root plus nodule N. Herbicide reduced the total plant nitrogen of O+N plants but it was still higher than for the other inoculation treatments where herbicide was applied. It can therefore be concluded that where no additional N was added, plant growth was nitrogen limited.

Herbicide treatment did not significantly affect the amount of N in the shoots or roots of O-N plants (Figure 6.4B). Unsprayed O+N plants had a greater amount of N in roots compared to all other treatments, but was significantly reduced by herbicide application. When herbicide was applied, all plants had the same amount of shoot and root plus nodule N except for the plus nitrogen treatment as explained above. Herbicide application reduced the amount of N in roots plus nodules for plants inoculated with straight RRI128 culture and the washed rhizobia compared with unsprayed plants. However herbicide application did not significantly reduce the root plus nodule N of plants inoculated with pre-exposed rhizobia.

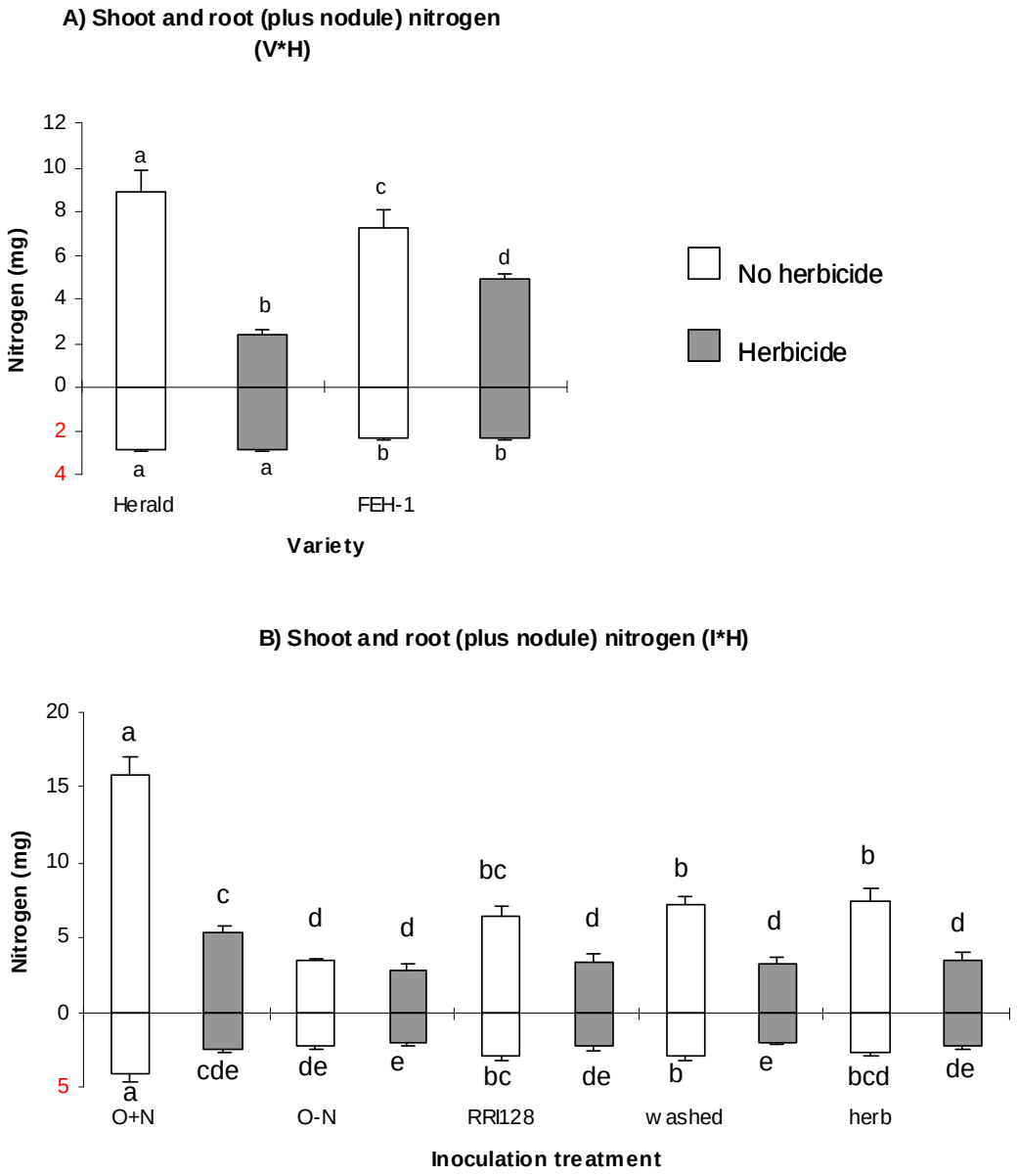

Figure 6.4 Plant nitrogen. Shoot nitrogen above the x-axis, roots plus nodules below the x-axis. (A) Variety by herbicide interaction for shoots ($p<0.001$, lsd5% = 0.938 g) and main effect of variety on roots plus nodules ($p<0.001$, lsd5% = 0.008 g). (B) Inoculation by herbicide interactions ($p<0.001$, lsd5% = 0.013 g). Error bars indicate standard error of mean.

For nitrogen partitioning, a variety by herbicide interaction was found, with near identical ratios to those observed in the dry matter partitioning (Figure 6.5 A). Chlorsulfuron increased the proportion of N in roots in Herald plants, but not in FEH-1 plants. There was also a significant inoculation by herbicide interaction for N partitioning (Figure 6.5 B). Herbicide application increased the proportion of N in

below ground organs for all inoculation treatments. Within the herbicide treatments, all inoculated plants had similar N distributions. For the uninoculated plants there was a lower proportion of N in roots where additional nitrogen was supplied. The proportion of N in roots of unsprayed O-N plants was higher than for any other unsprayed plants.

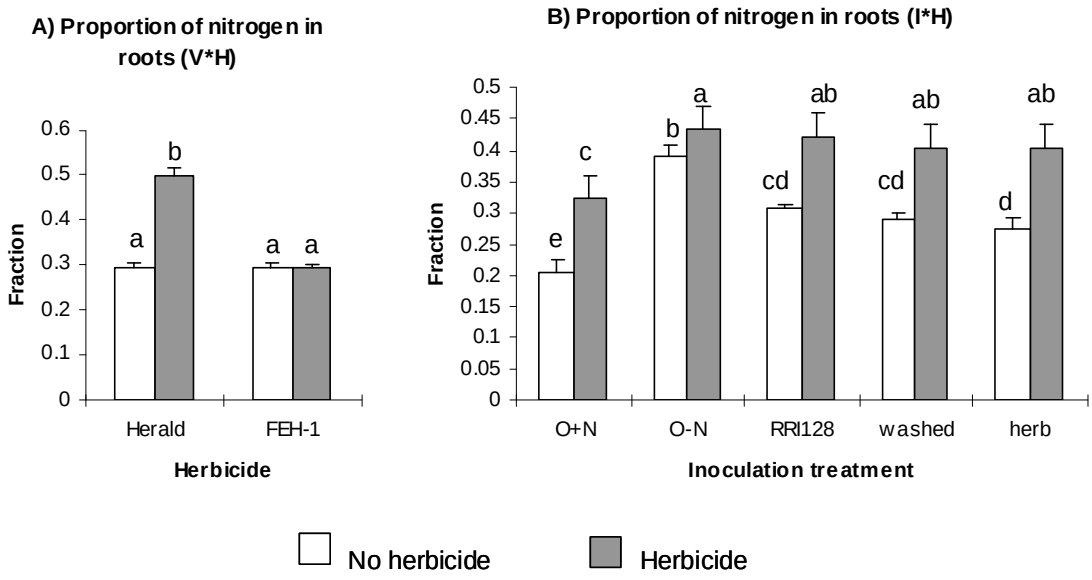

Figure 6.5 Partitioning of nitrogen between shoots and below ground (recovered roots and nodules) plant organs. (A) Variety by herbicide interaction ($p<0.001$, lsd5% = 0.023). (B) Inoculation by herbicide interaction ($p<0.001$, lsd5% = 0.036). Bars indicate standard error of mean.

6.3.3.3 Nitrogen concentration

The nitrogen concentration ([N]) was calculated for shoots, roots plus nodules, and whole plant samples. An inoculation by herbicide interaction affected total plant [N]. Herbicide application did not affect the [N] of O+N plants. However for O-N plants herbicide application resulted in a significant increase in [N]. Plants inoculated with straight RRI128 culture or washed rhizobia had higher [N] following herbicide application. When inoculated with rhizobia pre-exposed to herbicide, the plant [N] was unaffected by herbicide application to the plant (Figure 6.6 A).

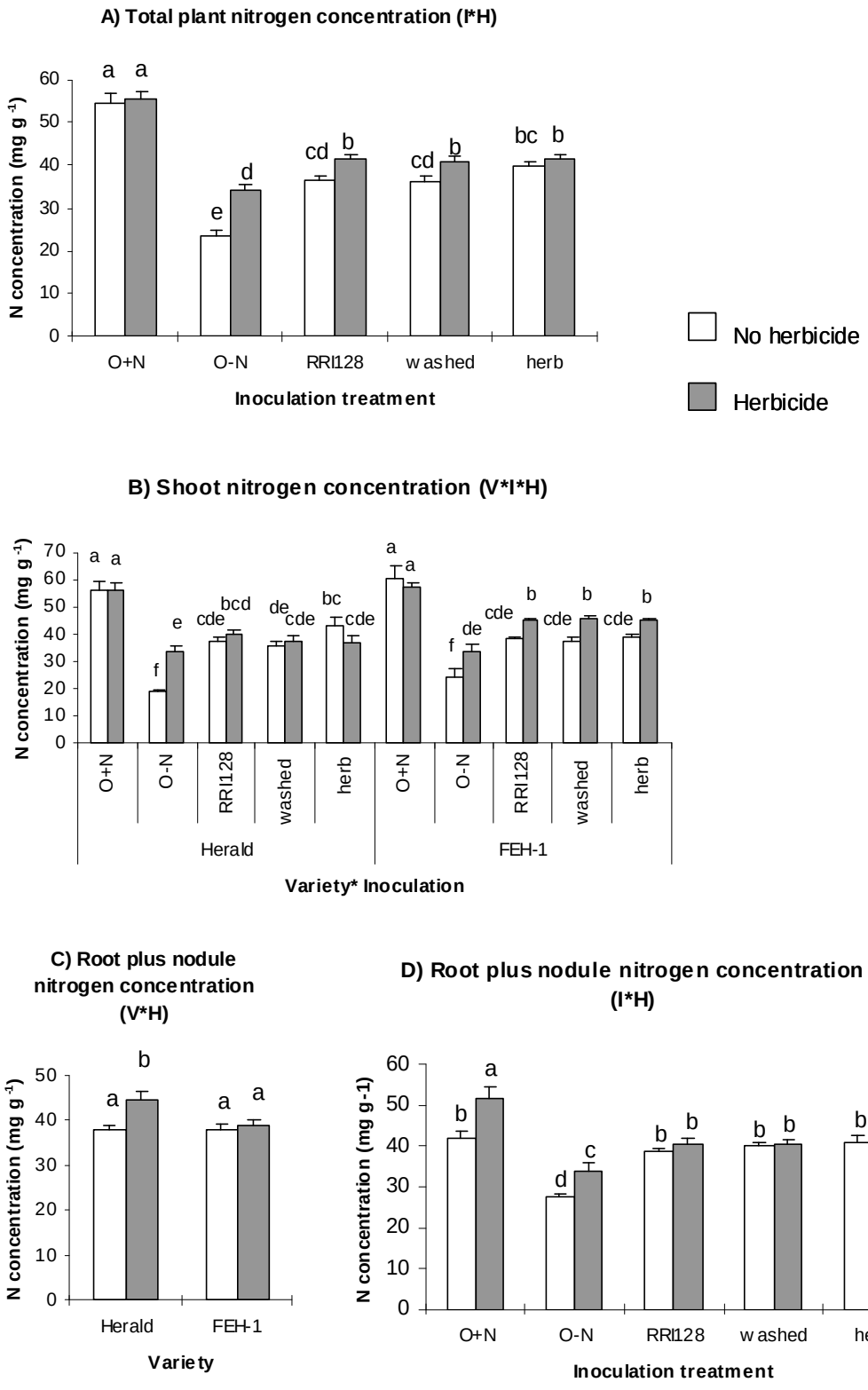

Figure 6.6 Plant nitrogen concentrations. (A) Inoculation by herbicide interaction on total plant nitrogen concentration (p = 0.005, lsd5% = 3.635) (B) Variety by inoculation by herbicide interaction affecting shoot nitrogen concentrations (p = 0.023, lsd5% = 6.122) (C) Variety by herbicide interaction (p = 0.002, lsd5% = 2.73) and (D) Inoculation by herbicide interaction (p = 0.019, lsd5% = 4.317) for below ground (roots plus nodules) nitrogen concentration. Bars indicate standard error of mean.

There was a significant 3-way variety by inoculation by herbicide interaction (p = 0.023) affecting shoot [N] (Figure 6.6 B). The major difference between varieties was that with application of herbicide, all inoculated FEH-1 plants had increased shoot [N] compared to the unsprayed plants. This contrasted with Herald plants, for which herbicide application caused no significant changes in the shoot [N] of inoculated plants. Amongst inoculated plants to which herbicide was applied, Herald plants had lower shoot [N] than FEH-1 plants. Within the unsprayed O-N plants, FEH-1 had a higher shoot [N] than Herald. Herbicide application did not change the shoot [N] of O+N plants of either variety.

Herbicide application elevated the [N] of roots plus nodules of Herald plants compared to unsprayed Herald plants and FEH-1 plants (Figure 6.6 C). There was also a significant inoculation treatment by herbicide treatment interaction (Figure 6.6 D). O-N plants had lower root [N] than the other inoculation treatments. Herbicide application increased the root [N] of uninoculated plants, but not the root plus nodule [N] of inoculated plants. O+N plants had similar [N] in the absence of plant applied herbicide to the inoculated plants, but significantly higher than the inoculated plants when herbicide was applied.

6.3.4 Nodulation

No nodules were observed on uninoculated plants indicating that no rhizobial contamination had occurred. Uninoculated plants were subsequently removed from the analysis of nodulation data, and a treatment structure of variety*rhizobia*herbicide used in the analysis of variance. Variety by herbicide interactions were observed for most variates, otherwise there was a main effect of herbicide (Table 6.4). An interaction involving the pre-exposure of rhizobia to herbicide was also observed.

Variety and herbicide treatments interacted to affect total nodule dry weight in a similar way to plant dry weight. In the absence of herbicide, Herald had a greater nodule dry weight than FEH-1. However herbicide application reduced nodule dry weight of Herald more so than FEH-1, so that after herbicide application, FEH-1 had a greater nodule dry weight than Herald (Figure 6.7 A).

Table 6.4 Significant main effects and interactions between variety, rhizobia and herbicide treatments on nodulation ($p<0.05$) as determined by analysis of variance. Where interactions are significant, the individual main effects are not presented. *Trends of interest are shown in parentheses.

Variable	Variety (V)	Rhizobia (R)	Herbicide (H)	Variety*Herbicide (V*H)	Rhizobia*Herbicide (R*H)	Variety*Rhizobia (V*R)	Variety*Herbicide*Rhizobia (V*H*R)
Nodulation (V*R*H)							
Total nodule dry weight				<0.001	*(0.087)		
Total nodule number				<0.001			
Total dry weight per nodule			<0.001				
Total nodule dry weight per unit root dry weight			<0.001	*(0.052)			
Total nodule number per unit root dry weight				<0.001			
Pink nodule dry weight				<0.001			
Pink nodule number				<0.001			
Dry weight per pink nodule			<0.001				
Pink nodule dry weight per unit root dry weight				<0.001		0.034	
Pink nodule number per unit root dry weight				<0.001			

For Herald plants, the number of nodules was reduced to a lesser extent by herbicide application than nodule dry weight (B). The number of nodules on FEH-1 plants was not significantly different with or without the CS. CS application reduced the dry weight per nodule from an average of 0.2 mg to 0.075 mg across the variety and rhizobia treatments, so not only did herbicide application result in fewer nodules, but on average the nodules were also smaller (Figure 6.7 C).

Pink nodule data (Figure 6.8 A and B) followed similar trends to the total nodule data. There were no significant effects of the rhizobia treatments on the dry weight of pink nodules. CS reduced the dry weight per pink nodule from 0.26 mg to 0.11 mg per nodule across the variety and rhizobia treatments (Figure 6.8 C).

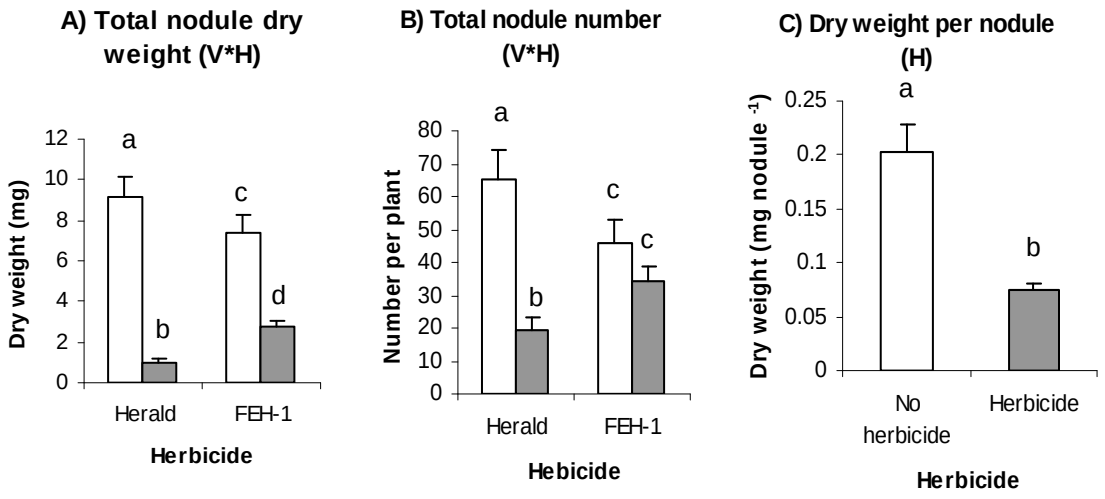

Figure 6.7 Nodulation. (A) Variety by herbicide interaction on the dry weight of nodules per plant ($p<0.001$, lsd5% = 1.22). (B) Variety by herbicide interaction on the number of nodules per plant ($p<0.001$, lsd5% = 13.1). (C) Main effect of herbicide on the dry weight per nodule ($p<0.001$, lsd5% = 0.072). Bars indicate standard error of mean.

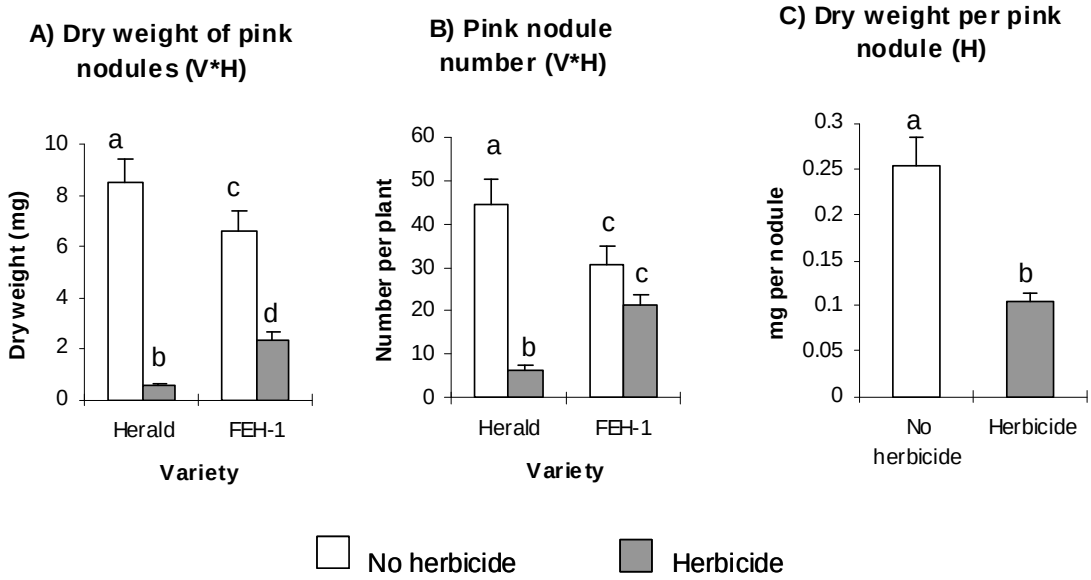

Figure 6.8 Nodulation. (A) Variety by herbicide interaction on the dry weight of pink nodules per plant ($p<0.001$, lsd5% = 1.167). (B) Variety by herbicide interaction on the number of pink nodules per plant ($p<0.001$, lsd5% = 9.81). (C) Main effect of herbicide on the dry weight per pink nodule ($p<0.001$, lsd5% = 0.084). Bars indicate standard error of mean.

Reductions in nodulation expressed on a root mass basis can indicate whether nodulation was affected to a greater extent than root growth. The dry weight of pink nodules per unit root dry weight of Herald was more severely impacted than FEH-1, resulting in a significant interaction between variety and herbicide treatments ($p<0.001$). In the absence of plant applied herbicide, FEH-1 and Herald had a similar pink nodule dry weight per unit root dry weight. However in the presence of plant applied herbicide, FEH-1 had greater nodulation on a root basis than Herald (Figure 6.9A). A similar result was seen for total nodule dry weight per unit root dry weight (data not presented).

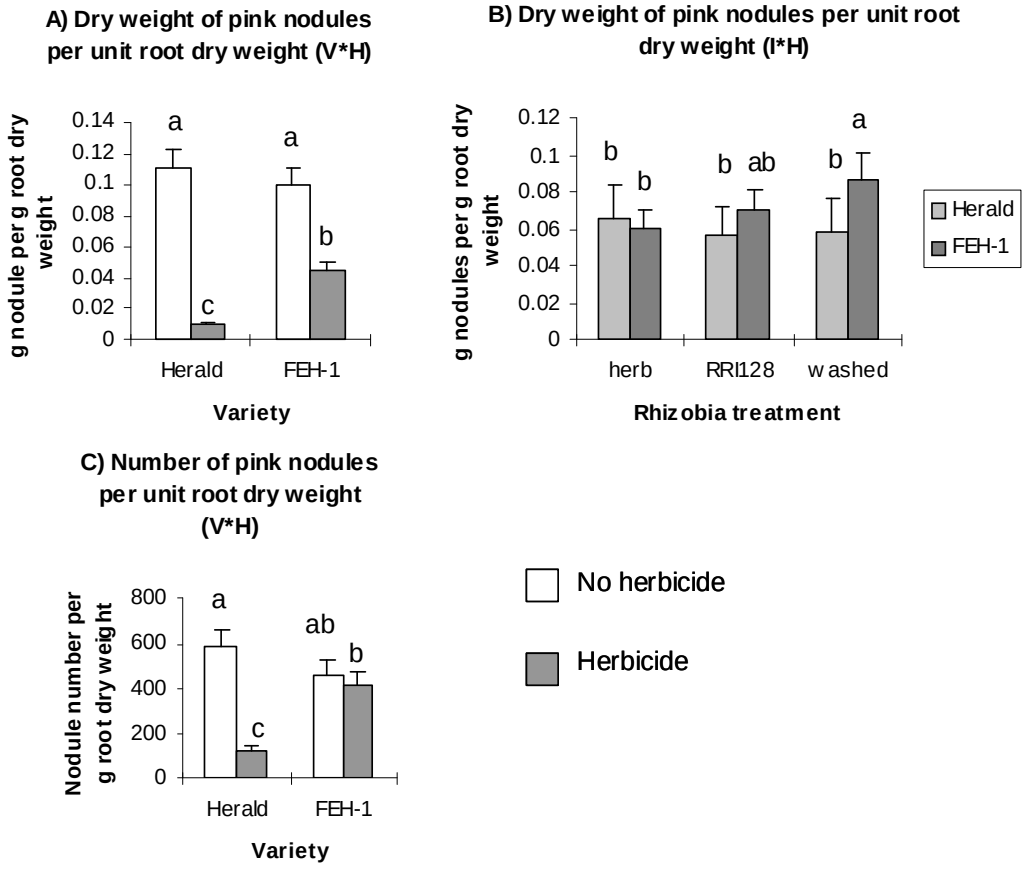

Figure 6.9 Pink nodule data expressed on a root dry weight basis. (A) Variety by herbicide interaction on the dry weight of pink nodules per unit root dry weight ($p<0.001$, lsd5% = 0.0147) (B) Inoculation treatment by herbicide interaction on the dry weight of pink nodules per unit root dry weight ($p=0.034$, lsd5% = 0.0180). (C) Variety by herbicide interaction on the number of pink nodules per unit root dry weight ($p<0.001$, lsd5% = 132). Bars indicate standard error of mean.

The rhizobia treatments affected the dry weight of pink nodules on a root dry weight basis differently for Herald and FEH-1 ($p=0.034$). Herald had similar pink nodule dry weight per unit root dry weight for all rhizobia treatments (Figure 6.9B). However for FEH-1, plants inoculated with herbicide-exposed rhizobia had significantly lower pink nodule dry weight per unit root dry weight than plants inoculated with washed rhizobia. Pink nodule dry weight per unit root dry weight was significantly higher for FEH-1 than Herald when inoculated with washed rhizobia, but not for the other rhizobia treatments. A similar pattern was seen for total nodule dry weight per unit root dry weight ($p = 0.052$, data not shown).

The number of pink nodules per unit root dry weight was unchanged for FEH-1 with or without plant-applied herbicide (Figure 6.9C). In the absence of herbicide, the mean number of pink nodules was higher for Herald than FEH-1. In the presence of herbicide, the number of pink nodules per unit root dry weight for Herald was severely reduced. A similar response occurred for total nodule number (data not shown).

6.3.5 Nitrogen Fixation

N_2 fixation at the time of harvest was assessed by the acetylene reduction assay, which provided an indication of the activity of the nitrogenase enzyme at the point in time when the plants were harvested. The amount of N_2 fixed over the duration of the experiment was calculated by the N difference method. No acetylene reduction activity (ARA) was detected in the uninoculated plants. Uninoculated plants were not included in further analysis of nitrogenase activity, and were only used for the calculation of the amount of N_2 fixed as described in the methods section.

Table 6.5 Significant main effects and interactions of variety, rhizobia and herbicide treatments on acetylene reduction activity and amount of nitrogen fixed ($p<0.05$) as determined by analysis of variance. *Trends of interest are shown in parentheses. Where interactions are significant, the individual main effects are not presented.

Variable	Variety (V)	Rhizobia (R)	Herbicide (H)	Variety*Herbicide (V*H)	Rhizobia*Herbicide (R*H)	Variety*Rhizobia (V*R)	Variety*Herbicide*Rhizobia (V*H*R)
Acetylene reduction assay (V*R*H)							
Acetylene reduction activity (ARA)				0.014			
ARA per g nodule dry weight			*(0.062)				
ARA per nodule	*(0.057)		<0.001				
ARA per g pink nodule dry weight							
ARA per pink nodule			<0.001				
Nitrogen fixation (V*R*H)							
Nitrogen fixed				0.012			
Nitrogen fixed per g nodule dry weight							
Nitrogen fixed per nodule			<0.001				
Nitrogen fixed per g pink nodule dry weight							
Nitrogen fixed per pink nodule			*(0.054)				

6.3.5.1 Acetylene reduction activity (ARA)

The impact of CS on the ARA of plants depended on the plant variety. Without herbicide, Herald and FEH-1 had similar ARA per plant. Herbicide application reduced Herald ARA to almost negligible levels, whereas FEH-1 was reduced to approximately one third of the FEH-1 control (Figure 6.10A).

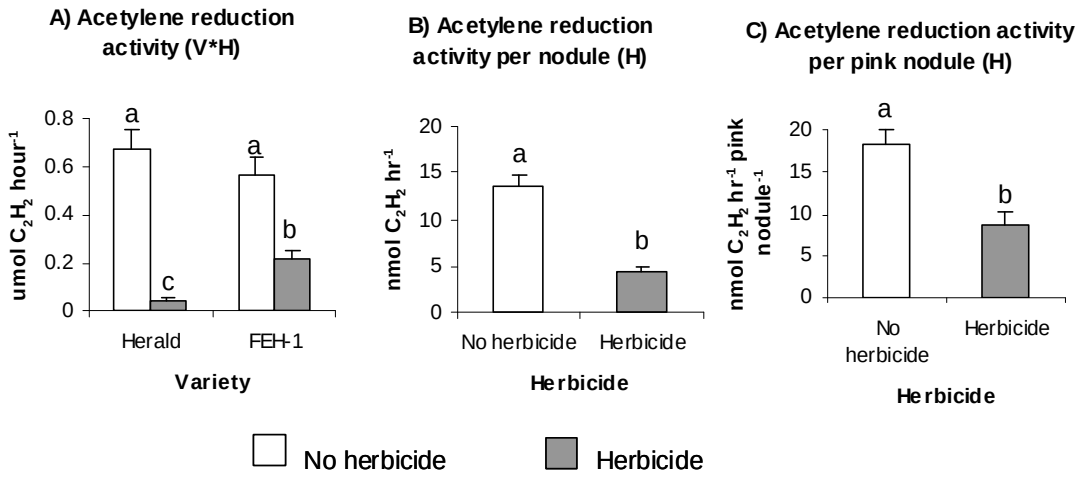

Figure 6.10 Acetylene reduction activity. (A) Variety by herbicide interaction on the ARA per plant (p=0.014, lsd5% = 0.143). (B) Main effect of herbicide on the ARA per nodule (p<0.001, lsd5% = 3.45). (C) Main effect of herbicide on the ARA per pink nodule (p<0.001, lsd5% = 5.47). Bars indicate standard error of mean.

Herbicide reduced ARA expressed on a total nodule number basis (Figure 6.10B) from 13.5 to 4.3 *n*mol C_2H_2 per hour per nodule for both varieties (p<0.001). No significant differences were observed when ARA was expressed on a pink nodule dry weight basis, indicating that there were no differences in the efficiency of the nodules that were pink. However when expressed per pink nodule (Figure 6.10C), CS reduced ARA by 52% across the two varieties (p<0.001). Although not statistically significant at a probability of 0.05, the data suggested that CS reduced ARA per unit nodule dry weight across the variety and rhizobia treatments from an average of 77.4 to 57.0 *n*mol C_2H_2 per hour per g nodule (p=0.062). Also ARA per nodule was higher for FEH-1 (10.6 *n*mol C_2H_2 per hour per nodule) than Herald (7.3 *n*mol C_2H_2 per hour per nodule) across the herbicide and rhizobia treatments (p= 0.057).

6.3.5.2 Nitrogen Fixation

The calculated amount of N_2 fixed per plant followed a similar trend to biomass and N data, with an interaction between variety and herbicide (p=0.012). Herald was found to have fixed more N than FEH-1 in the absence of herbicide. Plant applied herbicide reduced the amount of N_2 fixed by both varieties. Although the means indicated that Herald was affected more so than FEH-1, the difference between varieties in the presence of plant applied herbicide was not statistically significant (Figure 6.11A).

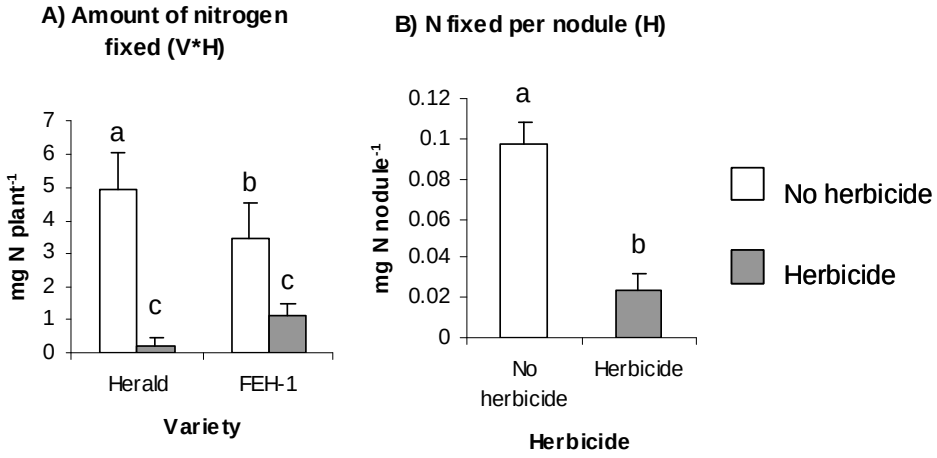

Figure 6.11 Amount of nitrogen fixed (A) Variety by herbicide interaction on the amount of nitrogen fixed per plant (p=0.012, lsd5% = 1.313). (B) Main effect of herbicide on the amount of nitrogen fixed per nodule (p<0.001, lsd5% = 0.038). Bars indicate standard error of mean

Plant applied herbicide reduced the calculated amount of N_2 fixed per nodule by 75% (p<0.001) (Figure 6.11 B). The amount of N_2 fixed per pink nodule decreased by 64%, (p = 0.054). There were no significant treatment effects on the amount of N_2 fixed per unit nodule dry weight.

6.4 Discussion

This experiment is the first assessment of the nitrogen fixing capability of FEH-1, a new herbicide tolerant cultivar of *Medicago littoralis*. The data provide further evidence that the impact of group B herbicides on nitrogen fixation is linked to the tolerance of the host legume. Reductions in plant dry weight, nodulation and nitrogen content were consistently greater in Herald than FEH-1, in accordance with the greater tolerance of FEH-1 to chlorsulfuron (Heap, 2000). However control Herald plants had significantly greater plant dry weight and nitrogen than control FEH-1 plants. These differences were not explained by differences in seed mass and nitrogen. Since FEH-1 did not display complete tolerance to the herbicide at the application rate used in this experiment, the relative contribution of the plant host and the rhizobia to reductions in N_2 fixation remain unclear. However there was little evidence to suggest that the rhizobia were affected by herbicide to the extent that it impacted on symbiotic nitrogen fixation.

6.4.1 General Plant Effects

The reduction in growth of uninoculated plants with and without additional N showed that at 0.75 g ha^{-1}, chlorsulfuron caused growth inhibition of both Herald and FEH-1, independent of N_2 fixation. The disruption of plant physiology that inhibited the growth of these uninoculated plants would also be expected to occur in symbiotic plants. The extent of inhibition of inoculated plant growth was dependent on the plant cultivar and hence the tolerance of the plant to chlorsulfuron (Figure 6.12). Thus the principle effects of herbicide in this experiment can be interpreted to be on the growth of the host plants.

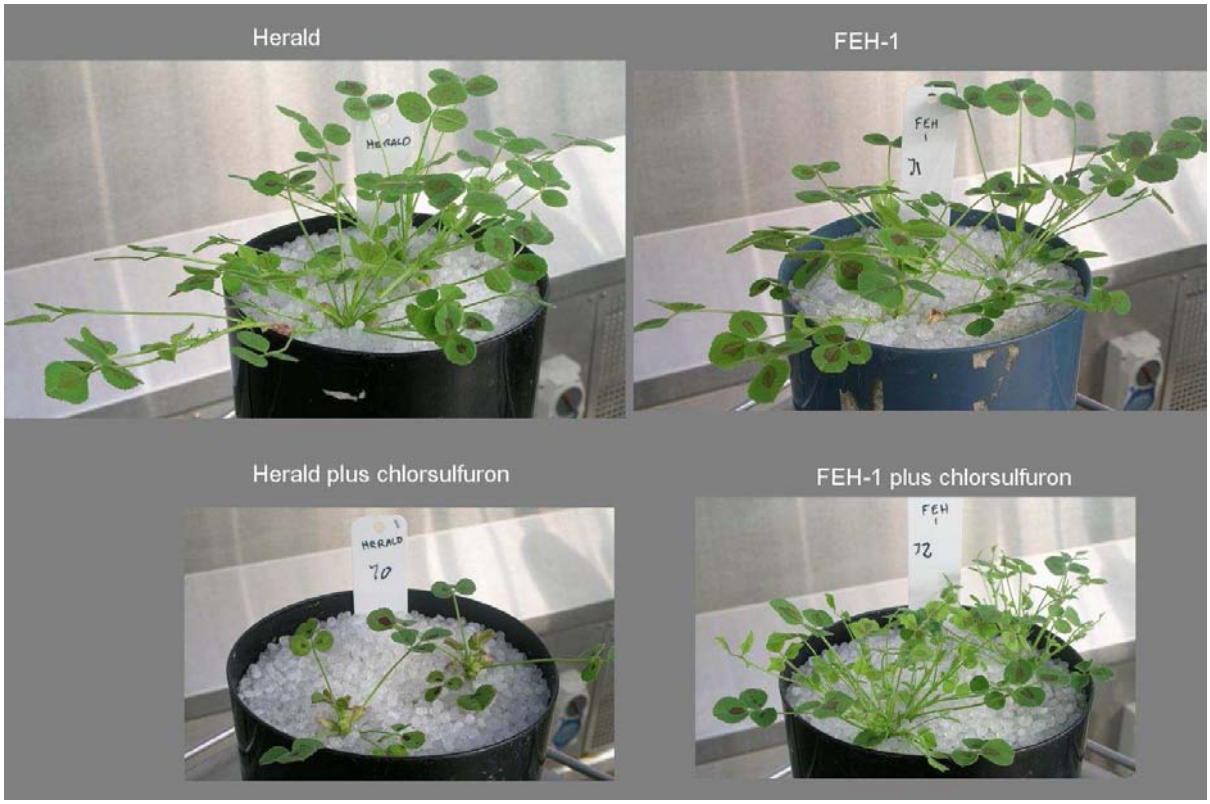

Figure 6.12 Effects of chlorsulfuron (bottom row) on the shoots of Herald (left) and FEH-1 (right) plants compared to zero herbicide controls (top row).

A number of the measured variates (plant dry weight, nitrogen, nodulation, ARA) were characterised by a similar variety by herbicide interaction. These treatment effects can be explained by the relative tolerance of FEH-1 to group B herbicides compared to Herald. CS at 0.75 g ha^{-1} decreased the total dry weight of Herald and FEH-1 plants by 64% and 34% respectively. In the presence of herbicide, FEH-1 had greater plant dry weight, nitrogen, nodulation and nitrogenase activity than Herald. However there may have been some penalty for the herbicide tolerance displayed by FEH-1, since Herald out-performed FEH-1 under herbicide-free conditions. Differences in seed nitrogen and carbohydrate stores did not contribute to these differences, as direct measurement of seed N by Kjeldahl analysis showed that FEH-1 seed had a slightly higher amount of N than Herald seed (0.168 mg and 0.140 mg per seed respectively, $p=0.003$). This was due mostly to higher seed mass (2.5 mg versus 2.2 mg, $p<0.001$) and possibly a higher [N] (6.61% versus 6.27%, $p=0.092$).

The amount of N in shoot and root samples generally followed the changes in biomass associated with variety, inoculation and herbicide treatments, though differences in the [N] were measured. Herald had a greater amount of N in roots plus nodules than FEH-1. For unsprayed plants, this was due to increased dry weight of below ground organs in Herald compared with FEH-1, considering that each cultivar had equivalent [N]. However, herbicide application reduced dry weight of below ground organs of both varieties to the same level. Where herbicide was applied, the higher amount of N in Herald roots plus nodules was related to an increase in [N], which may be explained by reduced demand for N from the stunted shoots, leading to an accumulation of N in the below ground organs.

In the experiment presented, CS was applied onto the shoots and the plastic beads on the soil surface. The reduction in root dry weight indicated that herbicide was translocated from shoots to roots by both varieties, or alternatively, some herbicide may have been washed into the potting mix and directly absorbed by the roots. Herbicide application did not change the partitioning of biomass and nitrogen for FEH-1, so the growth of roots and shoots of FEH-1 plants were equally inhibited. In contrast, foliar application of CS to Herald caused a far larger decrease in shoot dry weight than root dry weight, so a greater proportion of biomass and N was in the roots of Herald plants. The herbicide by variety interaction in the partitioning of biomass and N between shoots and roots suggested either that translocation of CS may have been different for Herald and FEH-1, or there was a more severe reduction of herbicide on the intolerant Herald shoots.

The inoculation treatments interacted with plant applied herbicide and variety to affect plant [N]. This was particularly apparent for shoot [N]. For uninoculated plants with a source of nitrogen in the potting mix (O+N), N was taken up from the growth medium. The cessation in shoot growth due to herbicide application was matched by an equal cessation in shoot N accumulation, hence there was no difference in [N] in the shoots compared to the zero herbicide plants. In contrast, herbicide did not alter the amount of N in the uninoculated plants with a limiting source of nitrogen (O-N). Therefore when herbicide reduced the growth of O-N plants, there was an increase in [N]. Amongst inoculated plants, the root and shoot [N] of Herald and FEH-1 responded differently. Herbicide application led to an increase in [N] in shoots of inoculated FEH-1 plants, but

no change to [N] of below ground organs. The herbicide might induce a more severe growth depression compared to the depression of nitrogen fixation or the reduction in N_2 fixation did not occur until some time after plant growth ceased. The excess N may have been translocated to the shoots resulting in an increase in shoot [N]. In contrast, CS did not affect [N] of shoots of inoculated Herald plants, but there was an increase in [N] of below ground organs. This might be indicative of a more abrupt disruption to translocation of fixed N in Herald plants.

The data clearly showed that group B herbicides affected plant growth, with most of the disruption related to the tolerance of the plant to herbicide. Plant effects were observed in uninoculated plants, also indicating that effects on nitrogen fixation were likely plant mediated. The increase in nitrogen concentration of FEH-1 shoots due to herbicide application suggested that plant growth was inhibited before the fixation and translocation of nitrogen was inhibited. The lack of such an effect in Herald might be related to the greater sensitivity of Herald and the severity of the response, or the tolerance mechanisms in FEH-1.

6.4.2 Growth of rhizobia *in vitro*

Previous studies involving exposure of rhizobia to group B herbicides have shown that there was no growth inhibition at concentrations of herbicide expected to occur in the field (Anderson *et al*., 2004; Eberbach and Douglas, 1989; Martensson and Nilsson, 1989). Similar results were found in this study for *Rhizobium meliloti* inoculum strain RRI128 at field application rates of herbicide. Addition of CS at concentrations of 15 *n*g ml^{-1} and 1.5 µg ml^{-1} slightly reduced the optical density of RRI128 cultures, but did not reduce the number of colony forming units counted.

As in Chapter 3 and 4 with flumetsulam, imazethapyr and imazamox, the results of the *in vitro* experiment with chlorsulfuron indicated that group B herbicides are unlikely to impact on the growth of rhizobia at commercial rates of herbicide application. However this is not to say that group B herbicides are benign to soil microorganisms (Ahonsi *et al*., 2004; Burnet and Hodgson, 1991; Forlani *et al*., 1995). The observed impacts of herbicide on the optical density of cultures (and hence possibly cell size since plate counts were not significantly different) would have been preceded by effects on cell function. The concentration of herbicide at which these more subtle changes begin to

occur remains undefined. It is important to consider the relevance of such experiments to saprophytic rhizobia in the soil, as discussed in Chapter 5. Broth cultures provide ideal conditions for growth, and might be expected to aid recovery of cells from metabolic perturbation caused by herbicide. Rhizobia in soil may not have the same ability to tolerate herbicide, and herbicide concentrations could be higher in the soil solution than in the bulk soil upon which the calculations were based to estimate liquid culture concentrations. Further incubation experiments at a range of herbicide concentrations followed by most probable number counts could address this issue.

6.4.3 Pre-exposure effects on rhizobia

The exposure of rhizobia to herbicide prior to inoculation affected shoot N concentrations, as well as total nodule dry weight and the dry weight of pink nodules per unit root dry weight in various interactions. Although these effects were small in magnitude relative to the impact of plant applied herbicide on many of the measurements, the pre-exposure effects may be indicative of alternative mechanisms by which group B herbicides inhibit N_2 fixation. They may also indicate a protective effect, where the relatively tolerant rhizobia assist the more susceptible host legume, as proposed by Royuella *et al*. (1998), however no such effect was found in this experiment.

For FEH-1, the dry weight of pink nodules per unit root dry weight was lower for herbicide treated rhizobia than washed rhizobia. However there was no difference for Herald. The data suggested that there was a 22% reduction in total nodule dry weight when unsprayed plants of both varieties were inoculated with herbicide pre-exposed rhizobia compared to the washed rhizobia treatment ($p=0.087$). Reductions in nodulation attributable to impacts of group B herbicides on rhizobia had not previously been reported until Anderson *et al*. (2004) in which exposure of rhizobia to CS reduced the nodule number and nodule dry weight of chickpea plants where there was no herbicide applied to the plant. In that situation, the nodules of plants inoculated with pre-exposed rhizobia compensated by fixing more N per unit nodule dry weight. Compensation had also been observed in the screening work in this thesis. Such compensation was not evident in this experiment.

The inclusion of a herbicide tolerant plant variety was expected to highlight any effects of herbicide on the microsymbiont. The lack of substantial effects of the pre-exposure treatment in this experiment suggests impacts on the rhizobia have only a minor role. However the incomplete tolerance of FEH-1 in this experiment means that some contribution of rhizobia to the intolerance of the symbiosis could not be discounted.

6.4.4 Nodulation

As well as the pre-exposure effects on nodulation noted above, nodulation was affected by plant applied herbicide following similar trends to shoot dry weight. Herbicide affected dry weight per nodule across both variety and all rhizobia treatments. Herbicide reduced the nodule dry weight per unit root dry weight for Herald and to a lesser extent FEH-1. By expressing nodule data on a root dry weight basis, it also appears that plant applied herbicide reduced nodule formation and development more so than root biomass. These reductions were attenuated by tolerance of the plant to chlorsulfuron, with nodulation per unit root dry weight less affected in FEH-1 than in Herald. Chlorsulfuron did not alter the number of pink nodules on a root dry weight basis for FEH-1 plants, so the reduction in pink nodule dry weight on FEH-1 plants was due to reduced development of these nodules. However for Herald, the 79% reduction in nodule number per unit root dry weight was accompanied by a 91% reduction in the dry weight of nodules per unit root dry weight. Hence in the more sensitive cultivar Herald, herbicide application reduced both nodule initiation (or increased nodule shedding) and nodule development. Thus the effects of herbicide on nodulation were related to the tolerance of the host plant.

As discussed in Chapter 5, reductions in nodulation can occur by shedding of existing nodules (Venkateswarlu *et al*., 1989), loss of viability of existing nodules (Matamoros *et al*., 1999), cessation of development of existing nodules (Sprout *et al*., 1992), or reduced initiation and development of new nodules after the time of herbicide application (Martensson, 1992). Since in this experiment chlorsulfuron actually decreased the number of non-pink nodules counted, it is unlikely that conversion of existing nodules to ineffective nodules was an important factor. It is possible that shedding of nodules occurred, however the reduction in the number of pink nodules per unit root dry weight in Herald plants may also be due to reduced infection and

nodulation caused by root hair deformation and disruption of meristems as observed in Chapter 7 and by Martensson and Nilson (1989).

Since nodule dry weight was reduced to a greater extent than the number of nodules, it could be interpreted that nodule development was more sensitive to the herbicide than nodule initiation. Whether this was directly due to herbicide stopping cell division or expansion of cells in the nodules, or a systemic response of the plant to herbicide and regulation of nodule development via long distance signalling (Gresshoff, 2003) from the shoots is unknown. Unpublished work (E. Drew, personal communication) on subterranean clover showed that nodule number reached a plateau by 17 days, whereas nodule dry weight continued to increase even at day 30. It is plausible that by the application of herbicide on day 18 in this experiment, most nodule initiation had already occurred. This could account for the greater reduction in nodule dry weight than nodule number found in this experiment. Gonzalez *et al*. (1996) found that imazethapyr applied pre-emergence to pea plants reduced nodule number more than size and so concluded that nodule initiation was more susceptible to group B herbicides. Similarly Martensson (1992) found application of CS to the rooting zone of red clover 4 days before inoculation with rhizobia resulted in significantly lower nodulation than when the herbicide was applied 4 days after the rhizobia. It was concluded that root hair deformations due to CS contributed to the lower nodulation. In this experiment, no measurements of plant and nodule dry weight and number were taken at the time of herbicide application, so a comprehensive comparison of the impact of chlorsulfuron on root and shoot growth versus nodule initiation and development cannot be made. However, it is probable that the impact of group B herbicides on nodulation is dependent on the time of application, and can affect nodule development as well as nodule initiation

Regressions of nodule dry weight and number against plant N can indicate how well the nodules have functioned over the duration of the experiment. Pink nodule dry weight was positively correlated with the total amount of N in plants. In the regressions between nodulation and total plant N, FEH-1 plants had higher total plant N than Herald plants at the low levels of pink nodule dry weight, but with increasing pink nodule dry weight, the increase in total plant N was greater for Herald than for FEH-1 (Figure 6.13

A). This can be explained by the relative tolerance of each cultivar to the herbicide, and might be indicative of better symbiotic compatibility or N_2 fixing efficiency in Herald plants. The Herald plants to which herbicide was applied clustered closer to the origin due to their sensitivity to CS. FEH-1 plants, with greater tolerance to the herbicide, formed a continuum throughout the range of nodule dry weights. At the higher nodule dry weights, unsprayed Herald plants had greater amounts of nitrogen per plant than the FEH-1 plants, where the proposed penalty for FEH-1's herbicide tolerance was maximised.

The regression between total plant N and pink nodule number (Figure 6.13 B) was best described by parallel lines grouped by the plant-applied herbicide treatments. With increasing nodule numbers, total plant N increased at the same rate independent of herbicide application. The lower y intercept for plus herbicide plants may indicate that herbicide reduced the ability of plants to take up N from the growth medium. If this was the case, this data suggest that the pink nodules function equally well, regardless of herbicide application.

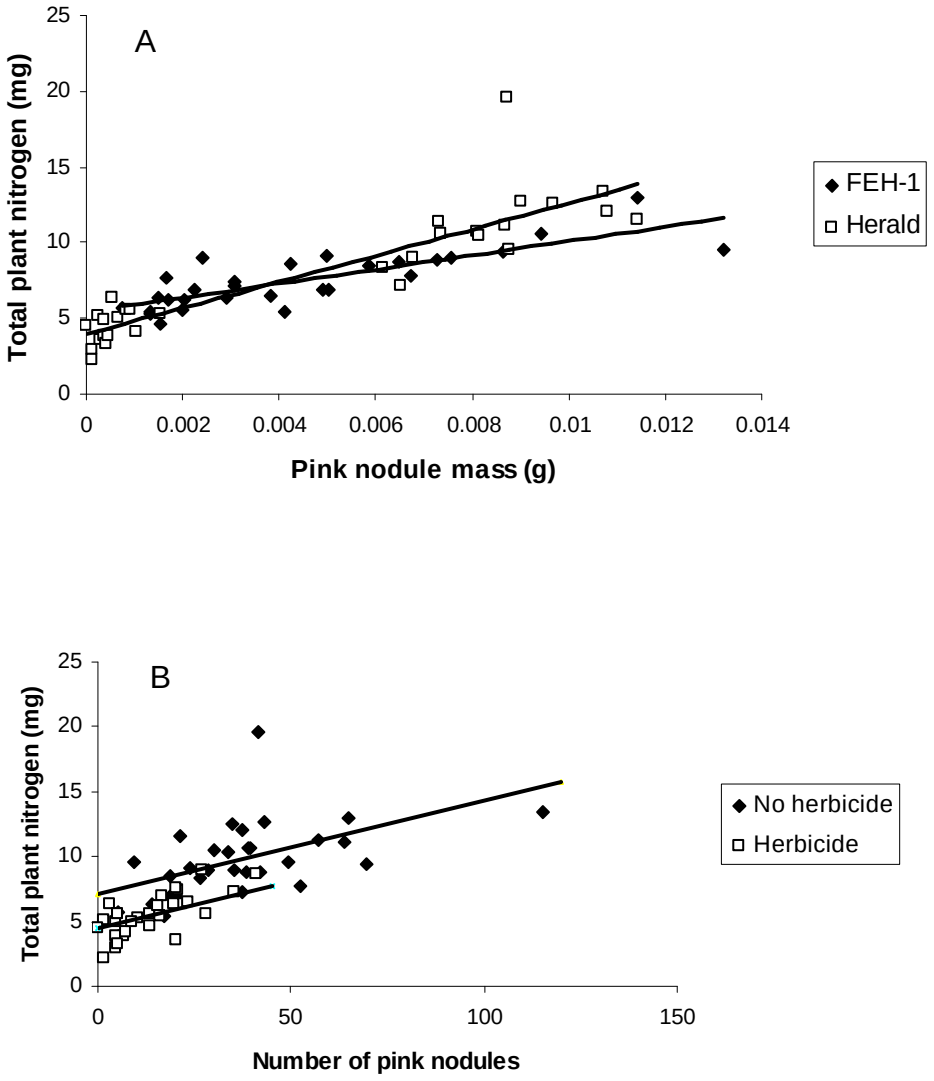

Figure 6.13 (A) Relationship between total plant nitrogen, and pink nodule dry weight of inoculated plants, grouped by variety. 76.4% of the variation accounted for. For FEH-1, the constant is 5.41 ($p<0.001$) and the regression coefficient is 469 ($p<0.001$). For Herald, the constant is 3.94 ($p<0.001$) and the regression coefficient is 866 ($p<0.001$). (B) Relationship between total plant nitrogen, and the number of pink nodules of inoculated plants, grouped by herbicide. Parallel lines with a slope of 0.071. Without herbicide, the constant was 7.16 ($p<0.001$), higher than with herbicide in which the constant was 4.50 ($p<0.001$). 61.2% of the variation accounted for.

6.4.5 Nitrogen fixation and acetylene reduction activity

This experiment represents the first characterisation of the N_2 fixing ability of FEH-1 compared to Herald. In the absence of herbicide, Herald fixed more nitrogen that FEH-1. Chlorsulfuron reduced the amount of nitrogen fixed in both Herald and FEH-1 cultivars. The reductions in the amount of N_2 fixed were more severe than effects on

plant dry weight. There was a clear reduction in the amount of N_2 fixed per nodule where herbicide was applied, however the amount of N_2 fixed per unit nodule dry weight was not significantly affected. These treatment effects were detected despite large variability in the data that might have been contributed to by the contaminating nitrogen source.

Acetylene reduction activity (ARA), an indicator of nitrogenase activity, was severely reduced in Herald plants by herbicide applied ten days previously. The ARA of FEH-1 plants was also reduced but to a lesser extent than for Herald. The acetylene reduction assay provided and indication of nitrogenase activity at a specific point in time (the end of the experiment in this study), whereas the nitrogen and biomass data measured the total amounts of nitrogen accumulated over the course of the experiment. Despite this, there were good relationships between ARA and plant N and biomass data.

There was a clear effect of herbicide on the relationship between shoot dry weight and acetylene reduction activity (Figure 6.14 A). Although herbicide application reduced the shoot dry weight, the relation to ARA had the same slope for both herbicide treatments. For higher ARA activities, the plant dry weight (Figure 6.14 B) and N (Figure 6.14 C) of Herald was greater than that of FEH-1. Point plots of shoot dry weight versus ARA clearly showed two separate data groups for Herald. Reduced production of photosynthate and hence energy supply for nitrogen fixation has been proposed as a mechanism by which nitrogen fixation is compromised (Schultze, 2004). Shoot dry weight was the best measure of photosynthetic capacity available in this experiment. Separate regressions for Herald and FEH-1 best described the relationship between ARA and shoot dry weight (Figure 6.14 B). This may provide further evidence that FEH-1 is symbiotically compromised, or perhaps that Herald was better able to access what little nitrogen was in the pots.

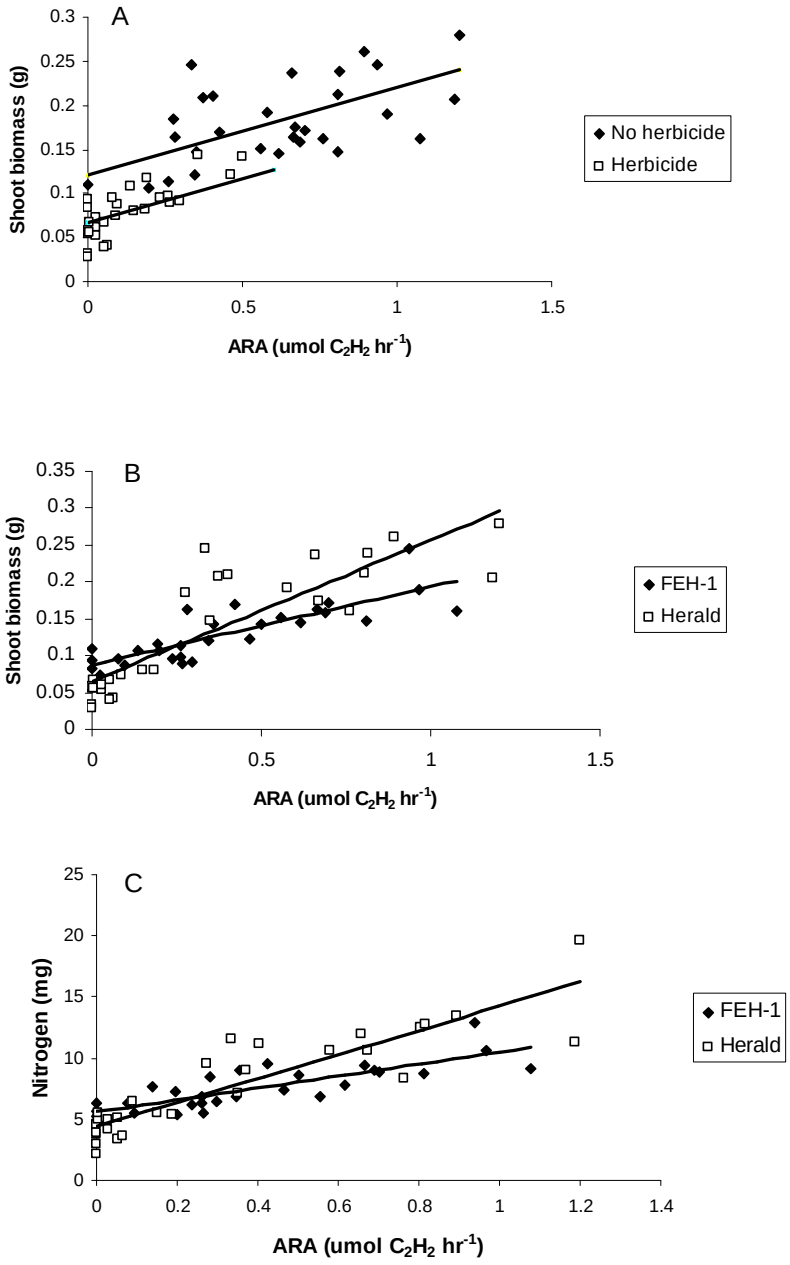

Figure 6.14 (A) Relationship between shoot dry weight and acetylene reduction activity, grouped by herbicide treatment. Parallel lines grouped by herbicide accounted for 76.5% of the variation. A common coefficient of 0.0988 ($p<0.001$), and constants of 0.1211 ($p<0.001$) for unsprayed and 0.0672 ($p<0.001$) for sprayed plants provided the best model. (B) Relationship between shoot dry weight and acetylene reduction activity, grouped by variety. Separate lines grouped by variety accounted for 72.8% of the variation. FEH-1 had a constant of 0.0875 ($p<0.001$) and a coefficient of 0.1058 ($p<0.001$), whereas Herald had a constant of 0.0655 ($p<0.001$) and a coefficient of 0.1913 ($p<0.001$). (C) Relationship between the total plant nitrogen, and acetylene reduction activity per inoculated plant, grouped by variety. For FEH-1, a constant of 5.6 ($p<0.001$) and a regression coefficient of 4.9 ($p<0.001$). For Herald, a constant of 4.4 ($p<0.001$) and a coefficient of 9.83 ($p<0.001$). 77% of the variation accounted for.

In contrast to shoot dry weight, there was not a good relationship between ARA and root plus nodule dry weight, with the regressions accounting for less than 50% of the variation. This may be due to a number of reasons. Firstly, it may be the case that shoot dry weight was more relevant to acetylene reduction activity in this experiment. For example, the photosynthate supply from the shoots might dictate the activity of the nitrogenase enzyme. The topical application of herbicide to shoots may mean that the impacts of herbicide on root dry weight were not as well correlated with the general effects on the plant and the symbiosis. This could be due to insufficient translocation of herbicide from shoots to the rest of the plant. There may also be control of N_2 fixation by long distance signalling in response to less demand for nitrogen in plant shoots that are not growing due to herbicide.

The relationships between ARA and pink nodule dry weight (Figure 6.15) or number (not shown) were disperse. There were no significant improvements to the regression of ARA against pink nodule dry weight after taking into account any of the treatments. However both pink nodule dry weight (Figure 6.13 A) and ARA (Figure 6.14 C) correlated well to total plant N despite the fact that ARA is a point measurement, whereas nodule dry weight and plant N both accumulated over time.

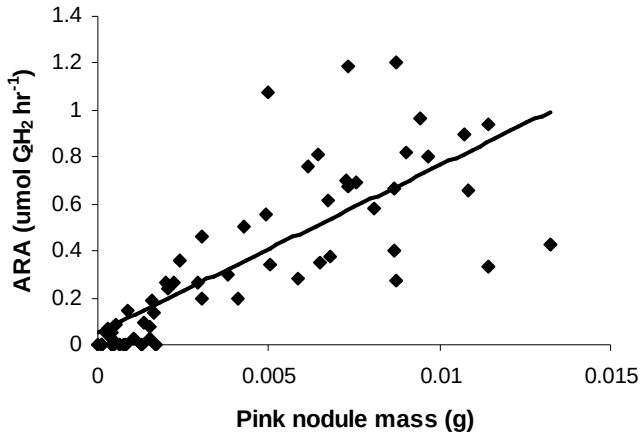

Figure 6.15 Relationship between acetylene reduction activity and the dry weight of pink nodules per plant. 60% of the variation, with regression coefficient (slope) of 71 ($p<0.001$) and a constant (y intercept) of 0.05 ($p=0.258$).

In addition to the significant results ($p<0.05$) discussed above, there were some interesting trends that add to the interpretation of the data. A possible decrease in ARA per unit total nodule dry weight due to herbicide application ($p=0.062$) could be due to the conversion of some of the effective nodule dry weight to ineffective nodule dry weight. FEH-1 seemed to have a higher ARA per nodule than Herald over the herbicide and rhizobia treatments ($p=0.057$), which would suggest that at the time of measurement, each FEH-1 nodule was more active. When expressed per pink nodule, herbicide application reduced ARA, consistent with the lower dry weight of these nodules.

6.4.6 Conclusions

The principle effect observed in this experiment was an inhibition of growth of the host plant by herbicide. FEH-1, the more tolerant of the two medicago varieties, had improved biomass, nodulation and nitrogenase activity over Herald when chlorsulfuron was applied at 5% of the application rate used in cereals. For Herald, there was a decrease in nodule number and nodule dry weight, even when expressed on a root dry weight basis. The more tolerant FEH-1 had reduced nodule dry weight per unit root dry weight, but the number of nodules per unit root dry weight did not decrease. Along with the reduction in dry weight per nodule over the variety and rhizobia treatments, these results suggest that nodule development, rather than initiation of nodules, was the primary effect of herbicide on nodulation, but further investigation is required to confirm this. Results of acetylene reduction assays indicated that reduced nitrogenase activity was also related to the susceptibility of the plant to herbicide.

In this experiment, FEH-1 did not display full tolerance to CS, and actually had reduced growth and nodulation compared with Herald in the absence of herbicide. For this reason, the experiment was not as mechanistically conclusive as was hoped. The relationship between the plant production variates, nodulation and acetylene reduction activity indicated that some discrepancies between the symbiotic performances of these varieties may exist. This may be related to a ‘tolerance penalty’ in FEH-1, whereby the growth and nitrogen fixation in the absence of herbicide was inferior to Herald.

The role of rhizobia in the susceptibility of symbiotic N_2 fixation to herbicide was limited, although minor changes to plant N concentrations and nodulation due to

exposure of rhizobia to herbicide prior to inoculation were measured. To ensure the success of herbicide tolerant varieties in rectifying reductions in N_2 fixation caused by group B herbicides, it would be prudent to undertake a more thorough assessment of the impacts of the herbicides on the rhizobia and their function. However it appears that FEH-1 will provide a useful alternative where the impact of residual and in-crop applications of group B herbicides on yield and N_2 fixation are of concern.

6.4.7 Implications for the use of herbicide tolerant legumes

Herbicides are an integral part of agriculture. Herbicide resistant crops have been pursued as a way of increasing the utility of existing herbicides. The selection of chemically mutagenised medicago plants for herbicide tolerance circumvents issues surrounding molecular methods of genetic modification, while developing a pasture legume that is tolerant of residues of group B herbicides, particularly the sulfonylureas, for use in areas where their persistence is known to be problematic. The intention was to develop a strand medic in which growth and hence biomass and yield were less affected by group B herbicides, and little attention had been given to the N_2 fixing capacity of this new cultivar – FEH-1.

In the work presented here the reduced N_2 fixation due to group B herbicides was principally linked to intolerance of the host legume to these herbicides. It is therefore likely that in a field situation where soil residues of group B herbicides exist, or where group B's are used directly on a pasture as part of a weed management strategy, FEH-1 would be a useful solution to reduced growth and N_2 fixation caused by these herbicides in the popular cultivar Herald. There is also the potential to create other herbicide tolerant legumes with similar benefits.

Of agronomic interest was an apparent 'tolerance penalty', in which Herald performed better than FEH-1 where no herbicide was applied. Howie and Bell (2005) compared shoot dry weight of Herald and FEH-1 in the field over a range of application rates of triasulfuron. At the zero application rate Herald had 3422 kg ha^{-1} compared with 3022 kg ha^{-1} for FEH-1. This had not been detected in other work on FEH-1, the difference being the nitrogen limiting conditions in this work, and more detailed analysis of the

symbiotic performance of the two varieties with and without a sulfonylurea herbicide. The nature of this 'penalty' deserves further attention.

6.4.8 Future work

More careful selection of a group B herbicide at a concentration at which FEH-1 is comprehensively tolerant might highlight further non-plant related mechanisms. An experiment involving triasulfuron, which has had extensive field testing in trials of FEH-1 and Herald (J. Howie, personal communication), would only require washed and pre-exposed inoculum, with uninoculated control plants. In the experiment described in this chapter, chlorsulfuron was applied onto the shoots of plants, which is more similar to an in-crop application of herbicide. The potential of herbicides to impact on nodulation may be greater when the active ingredient resides in the soil, as would occur where sulfonylureas persist into subsequent rotations. Such an effect needs to be characterised. Also, the experiment presented here was harvested between 10 and 14 days after herbicide application. Whether there is recovery or continued set-back in growth and N_2 fixation needs to be determined by extending the duration of the experiment. Multiple harvests will also decipher whether nodule initiation, development or shedding is most important in explaining the decreased nodulation caused by herbicide.

Further investigation into the effects of pre-exposure of rhizobia to herbicide may be warranted. Although these impacts were minimal in this experimental system, it is imperative that potential for herbicides to interfere with rhizobia in the field is characterised if there is to be certainty that the use of herbicide tolerant varieties will adequately address reductions in N_2 fixation.

Chapter 7 Morphology and proteome of *Medicago truncatula* roots in response to group B herbicides

7.1 Introduction

Group B herbicides can have a range of effects on the legume-rhizobia symbiosis. The mechanisms can be conceptually divided into two groups: 1) the impacts on the plant that can include secondary effects on root hair curling and flavenoid synthesis for example, which may have more obvious consequences for nodulation and subsequent nitrogen fixation; 2) impacts on the rhizobia and their survival as saprophytes, their ability to form symbioses, and their ability to function as bacteroids. The screening experiments showed that herbicide application at label rates can impact on plant growth,

nodulation and nitrogen fixation, with the extent of suppression varying among the herbicide / legume combinations studied. The comparison of Herald and FEH-1 varieties of *Medicago littoralis* showed that the impacts on nitrogen fixation were largely related to the tolerance of the plant to herbicide, and that plant intolerance appeared to be the principle way in which chlorsulfuron impeded nitrogen fixation. In this chapter the impact of group B herbicides on legumes is further characterised, with the aim of exploring the mechanisms by which N_2 fixation might be inhibited via herbicide effects on the plant.

Although group B herbicides are known to inhibit the acetohydroxyacid synthase enzyme in the path of branch chain amino acid synthesis, the exact mechanisms of plant death have not been satisfactorily determined (see discussions in earlier chapters). It is therefore difficult to prioritise areas of study to focus on when considering the potential impacts of group B herbicides on nitrogen fixation. Proteomics is the systematic analysis of the proteome of cells, tissues or organisms. The proteome is the full complement of all proteins, including enzymes, transporters, and regulatory proteins, under a given set of conditions. Two dimensional gel electrophoresis of extracted proteins combined with gel analysis and subsequent identification of proteins by mass spectrometry allows the comparison of proteomes under different experimental treatments, and the identification of differentially expressed proteins. Proteomics therefore allows a more 'global' approach to addressing biological questions.

The Genomics Interactions Group within the Research School of Biological Sciences (RSBS) at the Australian National University have established proteomics capability for the study of the model legume *Medicago truncatula* cv A17 (Mathesius *et al*., 2001). Few studies have used proteomics to examine the impacts of herbicides on plants. Of the limited work relating to this area, a number of proteins induced by the safener fluxofenim were identified in a proteomic analysis of wheat coleoptiles (Zhang and Riechers, 2004). These included a number of glutathione s-transferases, along with stress response and glycolysis proteins. Teixeira *et al*. (2005) used proteomics to examine the response of yeast as a model eukaryotic organism to the herbicide 2,4-dichlorophenoxyacetic acid.

Experiments were conducted to examine the impact of group B herbicides metsulfuron methyl and flumetsulam on root growth, root hairs and lateral root formation, as well as a proteomic analysis of the root tips of *Medicago truncatula* A17 grown on agar plates. Root tips contain the root apical meristem which produces cells for all of the tissues of the main root. Meristems are important sites for herbicide inhibition of growth and are also required for the formation of nodules. Meristems are the site of herbicide accumulation, where initial symptoms of group B herbicides are observed and where necrosis first occurs (Duggleby and Pang, 2000). This work was aimed at identifying cellular processes and functions that are disrupted or enhanced by group B herbicides using a proteomic approach. The intention was to increase our understanding of how these herbicides function, and identify effects on the plant that may compromise the development of effective symbioses.

7.2 Methods

7.2.1 Plant growth

Medicago truncatula cv. A17 seeds (a volume of 7.5 ml per experiment) were sterilised in 6.25% sodium hypochlorite solution with shaking for 5 minutes. The seed was then rinsed 6 times in sterile water, prior to placement on 9 cm plates containing Fåhreus medium ($CaCl_2.2H_2O$ (132 mg L^{-1}); $MgSO_4.7H_2O$ (120 mg L^{-1}); KH_2PO_4 (100 mg L^{-1}); $Na_2HPO_4.12H_2O$ (150 mg L^{-1}); ferric citrate (5 mg L^{-1}); H_3BO_3 (2.86 mg L^{-1}); $MnSO_4.4H_2O$ (2.03 mg L^{-1}); $ZnSO_4.7H_2O$ (0.22 mg L^{-1}); $CuSO_4.5H_2O$ (0.08 mg L^{-1}); $H_2MoO_4.H_2O$ (0.09 mg L^{-1}); 0.8% agar). One ml of sterile water was added to each plate to ensure adequate moisture for germination. The plates were then sealed with Nescofilm, wrapped in foil and kept at 4°C for at least 24 hours to synchronise subsequent germination of the seed. Plates were placed vertically in a dark incubator set to 27°C, so that radicles grew unidirectionally.

After 24 to 48 hours, 10 germinated seedlings were carefully transferred to each of thirty 15 cm Fåhreus media plates containing either filter sterilised technical grade flumetsulam (Dow AgroSciences Australia) at 200 *n*g per ml, metsulfuron methyl (DuPont Australia) at 10 *n*g per ml, or no herbicide. A sterile aluminium foil spacer was placed at the top of each plate to allow gas exchange. The bottom two-thirds to three-quarters of each plate was sealed with Nescofilm. Plates were placed vertically in

wire trays, with black cardboard between plates to reduce the amount of light reaching the roots.

Plates were incubated at 350 μmol $m^{-2}s^{-1}$ photons light intensity with 16 hour day, constant 21°C, and 86% relative humidity for 5 days for protein samples, up to 18 days for root length measurements and morphological observations. Experiments were repeated three times for the protein samples.

The positions of the root tips of 70 seedlings for each treatment were marked on the back of the agar plate at 3, 6, 9, 12 and 18 days. At the end of the experiment, plates were scanned on a flatbed scanner and root lengths measured using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA). Cumulative root lengths and relative growth rates were calculated from day 3, to avoid initial differences in radical lengths and time taken for growth to initiate following transplanting. The numbers of lateral roots on 18 day old plants were also counted off these scans.

During the experiment, observations of whole roots were made under a dissecting microscope. Roots were also stained in methylene blue to observe vascular structures and highlight the meristematic regions.

7.2.2 Root harvest and protein extraction

Three separate experiments were set up as described above to obtain roots for proteomic analysis. In each experiment, 25 plates of 10 plants per plate were harvested for each treatment. A glass slide was placed over a template with 0 mm, 3 mm and 10 mm demarcated. Five day old plants were removed from their plate, lined up on the slide, and sections 3 mm from the tip, and a further 10 mm along the root were cut and placed in pre-weighed Eppendorf tubes in liquid nitrogen. The samples were then weighed and stored at -80ºC until ready for protein extraction.

To extract protein from the root samples, frozen plant tissue for each treatment (herbicide*root section) was ground with glass beads in a stainless steel mortar kept on liquid nitrogen, and a ceramic pestle. When ground to a fine powder, the sample was

transferred to an acetone resistant centrifuge tube on dry ice containing 10 ml of freshly prepared extraction solution (10% trichloroacetic acid (TCA) and 0.07% dithiothreitol (DTT) in acetone) per gram of sample. This was sonicated 6 times for 10 seconds with 60 second intervals in dry ice. Samples were then left for at least 1hour at –20ºC.

Tubes were centrifuged in a SS-34 rotor for 15 minutes at 17 000 rpm and 4ºC. The supernatant was removed, and the pellet crushed in 10 ml of washing solution (0.07% DTT in acetone). This was left on dry ice for 30 minutes before centrifuging at 10 000 rpm at 4ºC for 15 minutes. Washing was conducted 3 times, transferring the resuspended pellet to Eppendorf tubes before the last spin. Pellets were then dried in a laminar flow hood for a few hours.

Pellets were solubilised by crushing with approximately 50 µl of solubilisation buffer (9 M urea, 4% (w/v) CHAPS (Bio-Rad), 1% DTT (Hercules), 1% (w/v) Biolyte® 3/10 Ampholyte pH 3-10 (CA), 35 mM Tris Base in MQ water) per 10 mg of dry pellet. Samples were vortexed for 30 seconds, then sonicated in a water bath for 30 seconds five times. Tubes were then centrifuged at 12 000 rpm at room temperature for 15 minutes. The supernatant was removed to freshly labelled Eppendorf tubes. Solubilisation of the pellet was repeated and the supernatant added to the first lot of transferred supernatant.

A 3ul aliquot of solubilised protein was removed for determination of the protein content by the Bradford assay. This was diluted 1 in 10, then 1 in 2 in solubilisation buffer (see above) to give final dilutions of 1 in 10 and 1 in 20. Along with bovine serum albumin standards to 1.2 mg ml^{-1}, 5 µl duplicates of protein sample were mixed with 245 µl Bradford dyes (Bio-Rad) in an ELISA plate. The plate was read at 595 *n*m, and protein concentrations calculated.

7.2.3 Gel electrophoresis

For each treatment in each of three experiments, 200 µg of protein was cup loaded onto rehydrated Immobiline DryStrips with pH 4-7 (Amersham) for first dimension separation of proteins by isoelectric point. Second dimension gels were run using

precast ExcelGel SDS gels with a 12-14% acrylamide gradient on a Multiphor II horizontal electrophoresis system.

7.2.4 Gel staining

Gels were silver stained for comparing proteomes of the treatments. Gels were fixed 3 times for 15 minutes in 250 ml of 40% ethanol and 10% acetic acid. They were then sensitised overnight (30% ethanol, 2.5 g potassium tetrathionate, 68 g sodium acetate trihydrate, 20 ml of 25% glutaraldehyde made to 1 L). Gels were then rinsed with 1 L milliQ water 6 times for 15 minutes. Staining was for 2 hours in 250 ml staining solution (2 g silver nitrate, 0.623 g HEPES, 700 µl 37% formaldehyde made to 1 L). After a 10 second rinse in milliQ water, gels were developed for between 4 and 12 minutes until good resolution was observed. The developing solution consisted of 30 g potassium carbonate, 10 mg sodium thiosulfate pentahydrate, 250 µl of 37% formaldehyde solution, made to 1 L with milliQ water. Development was stopped for 10 minutes in 25 g Tris base and 25 ml acetic acid made to 1 L. All steps were conducted on a tilting platform and solutions discarded appropriately prior to the next step. Gels were sealed in plastic bags and stored at 4°C until scanning and analysis.

7.2.5 Gel analysis

Silver stained gels were scanned at 300 dots per inch on a UMAX Astra 2400S flatbed scanner (UMAX Technologies, Freemont California), and imported into Melanie 4 software (Swiss Institute of Bioinformatics, Geneva, Switzerland). There were 3 separate experiments, with 3 herbicide treatments and 2 root sections, giving a total of 18 gels for analysis.

A gel matching procedure was conducted, in which ‘landmark’ spots present on all gels were manually identified, and the gel matching procedure of ImageMaster software (GeneBio, Geneva, Switzerland) subsequently used to match spots on gels one pair at a time. This was checked and a few missing spots were identified manually before running the gel matching procedure again, until most spots were matched. Individual identities were then assigned to the spots. Every spot on each repeat gel of treated

samples was compared to each repeat gel of the control samples, as indicated inFigure 7.1

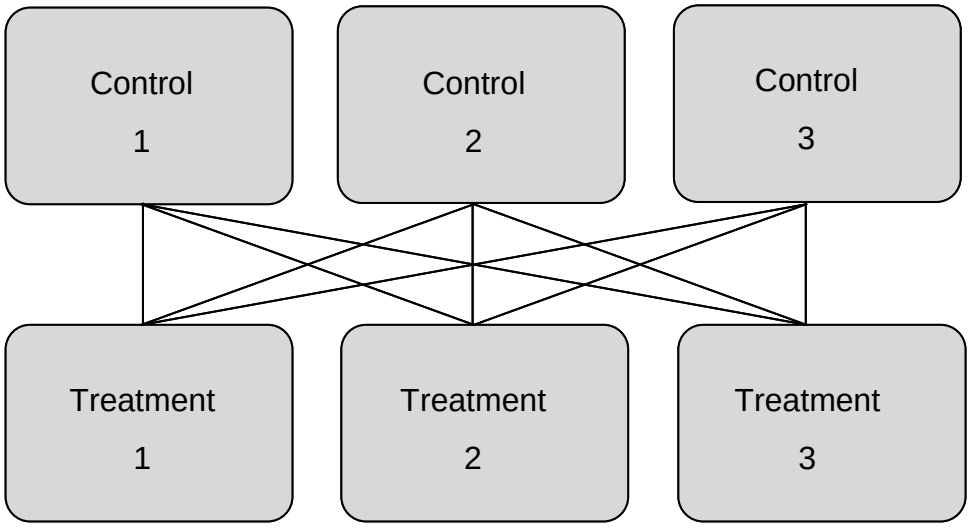

Figure 7.1 Schematic of gel matching procedure by Imagemaster

The volume of each assigned spot is a function of its size and intensity as measured by the Melanie 4 software.

The percent volume (%Vol) was calculated for each protein spot as follows:

$$\%Vol = \frac{Vol}{\sum_{s=1}^{n} Vol_s} * 100$$

The use of %Vol accounts for gel to gel variation in staining and protein loading. It provides the relative abundance of a particular protein compared to the entire complement of proteins from the sample.

7.2.6 Gel Statistics

Treatment effects on the accumulation of individual proteins were determined by Restricted Maximal Likelihood (REML) analysis using Genstat 8.0 (VSN International, United Kingdom). This procedure allows the analysis of unbalanced data. Where p values were less than 0.05, the least significant difference (LSD) was calculated and the means compared.

7.2.7 Protein identification

Where spots could not be identified by matching to the protein reference map of the *M. truncatula* root or embryogenic culture (Imin *et al.*, 2004; Imin *et al.*, 2005; Mathesius *et al.*, 2001), they were identified by mass spectrometry.

One mg of pooled protein sample was run on a two dimensional gel as described above, and the gel stained with Coomassie Brilliant Blue. To make the staining solution, 100 g ammonium sulphate, 1 g Coomassie Brilliant Blue, 340 ml methanol and 24 ml of 85% phosphoric acid were made to 1 L, and 250 ml used to stain the gel overnight on a tilting platform. Destaining solution was prepared by dissolving 12.1 g Tris base in 500 ml milliQ water, and adjusting the pH to 6.5 with phosphoric acid, before making to 1 L with milliQ water. Gels were washed in destaining solution for 2 minutes, then 25% methanol for 1 minute. Gels were then washed for 12 hours in 20% ammonium sulphate. The entire staining procedure was repeated, and the gel stored in 20% ammonium sulphate at 4°C.

Spots of interest were excised from the Coomassie stained gel using a clean scalpel blade, and placed into separate Eppendorf tubes. These were incubated with 120 µl of destaining solution (50% acetonitrile, 25 mM NH_4HCO_3, pH 7.8) at 37ºC for 10 minutes, with periodic shaking. This was repeated with fresh destaining solution until the gel pieces were clear. Gel pieces were then dried in a Speed Vac for 10 minutes on low setting.

Each protein sample was digested for 16 hours with 8 µl of trypsin solution (15 *n*g ul^{-1} sequencing grade trypsin from Promega, 50 mM NH_4HCO_3, pH 7.8) at 37ºC. Peptides were acidified by adding 1% triflouroacetic acid (TFA) (pH < 4) and were then extracted from the gel pieces using ZipTipC18 Reverse-Phase pipette tips (Millipore, Bedford, MA). The peptides were eluted in 0.1 % TFA for MALDI-TOF-TOF MS/MS analysis or in MeOH and 0.1% formic acid for LC MS analysis.

For matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF), fresh matrix was prepared by vortexing α-cyano-4-hydroxycinnamic acid (8 mg ml^{-1}) in solvent (70% v/v acetonitrile and 1% v/v TFA in milli-Q water) to form a saturated solution. This was centrifuged and 1 µl of the supernatant spotted onto

a 7x7 place target. 0.5 µl of digested protein sample was spotted onto the matrix and allowed to air dry.

MALDI mass spectrometry was performed with an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in MS mode. Following MALDI-TOF, the instrument was then switched to MS/MS mode, and the eight strongest peptides from the MS scan were isolated and fragmented by collision-induced dissociation with filtered lab air, then re-accelerated to measure their mass and intensities. The data was exported in a format suitable for analysis with Mascot (Matrix Science, London). LC MS/MS data acquisition was performed with a LCQ DECA XP Plus (Thermo Electron, San Jose, CA) and data exported in a format suitable for analysis with Mascot. Peptide mass fingerprinting (PMF) was done on a Micromass TofSpec 2E time of flight mass spectrometer (Waters, Milford, MA, USA).

Searches were run against the *M. truncatula* gene index database (MtGI, Release 8.0, January 2005) that contains ~37,000 minimally redundant tentative clusters (TC) of *M. truncatula* expressed sequence tags (EST) downloaded from The Institute of Genomic Research (TIGR) (ftp://ftp.tigr.org/pub/data/m_truncatula/). MS and MS/MS data were analysed using Mascot (Matrix Science, London) to search the MtGI database, for which MOWSE scores (Pappin *et al*., 1993) greater than 66 are significant ($p< 0.05$).

Searches were conducted using a mass accuracy of ±25 ppm and no missed trypsin cleavages; this was changed to ±100 ppm and one missed cleavage when no significant matches were found. In the searches the following mass modifications were allowed: carbamidomethyl modification of cysteine residues by iodoacetamide, oxidation of methionine, and propionamide modification of cysteine by acrylamide. For peptide mass fingerprinting matches with significant Mowse scores (Pappin *et al*., 1993), 4 peptides are sufficient to predict a statistically significant match. For MS/MS, data matches with fewer peptides were also examined. Proteins were subjected to a protein-protein BLAST search against the National Centre for Biotechnology Information non-redundant protein database to validate that the correct open reading frame had been predicted by TIGR. Matches were further validated by comparing relative molecular weight and isoelectric point calculated from 2 dimensional electrophoresis with that of the matching protein.

The gel matching and statistics and the protein identification was described and completed by Peta Holmes, Research School of Biological Science, Australian National University (Holmes *et al*., 2006).

7.3 Results

7.3.1 Root growth and morphology

Metsulfuron methyl had more severe impacts on root morphology than flumetsulam, however a common sequence of morphological responses was evident. The impacts of both herbicide treatments on root gross morphology were first evident in the reduced density and length of root hairs compared to controls. Control plants had a dense covering of root hairs, uniformly tapering towards the root tip (Figure 7.2 A and D). Patchy root hair distribution was observed on herbicide treated plants, with some areas of excessive root hair density. However the majority of aberrant areas had sparse root hair density (Figure 7.2 B,C, E and F).

Medicago truncatula plants grown on plates containing agar with flumetsulam and metsulfuron methyl had lower rates and amounts of root growth and altered root morphology compared with control plants. Measurement of primary root lengths (Figure 7.3) from 3 days after transplant, and calculation of relative growth rates showed that by nine days after transplanting, metsulfuron methyl treated roots grew slower than controls, and ceased growing between 12 and 15 days after transplanting to herbicide plates. At day 15, flumetsulam treated primary roots were shorter than controls. Repeated measures ANOVA of cumulative root lengths showed a significant time*treatment interaction ($p<0.001$).

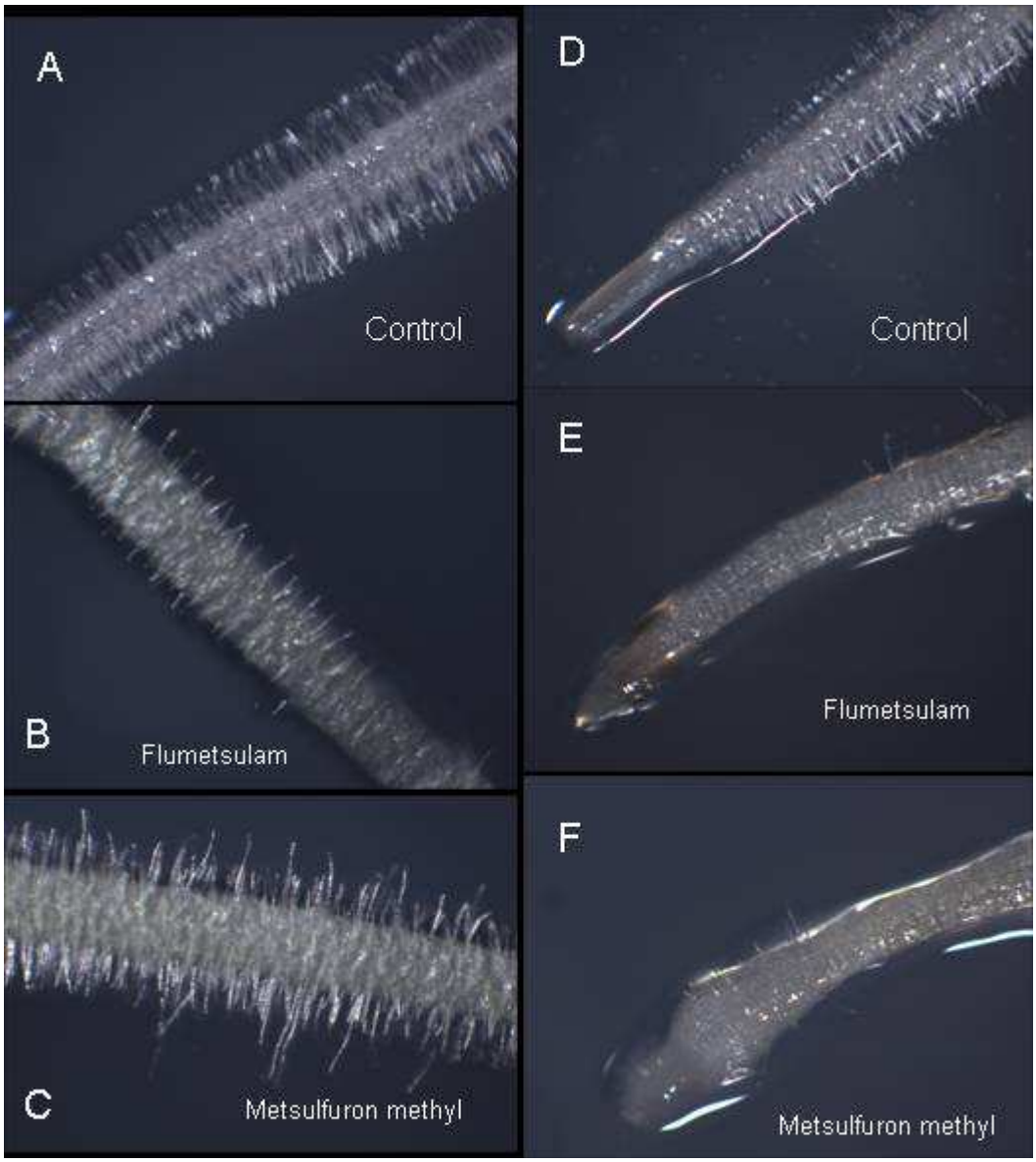

Figure 7.2 Root tips (D, E and F) adjacent zones (A, B and C) of 6 day old *Medicago truncatula* A17 grown on agar plates with no herbicide (Control: A and D), flumetsulam (B and E) or metsulfuron methyl (C and F). All plants grown on control plates had uniform distribution of root hairs, with lengths tapering towards the tip. Both herbicide treatments caused erratic root hair distribution and irregular root hair lengths. Note the sparse root hairs on herbicide treated roots, and the deformed 'bulbous' root tip on the metsulfuron methyl treated plant.

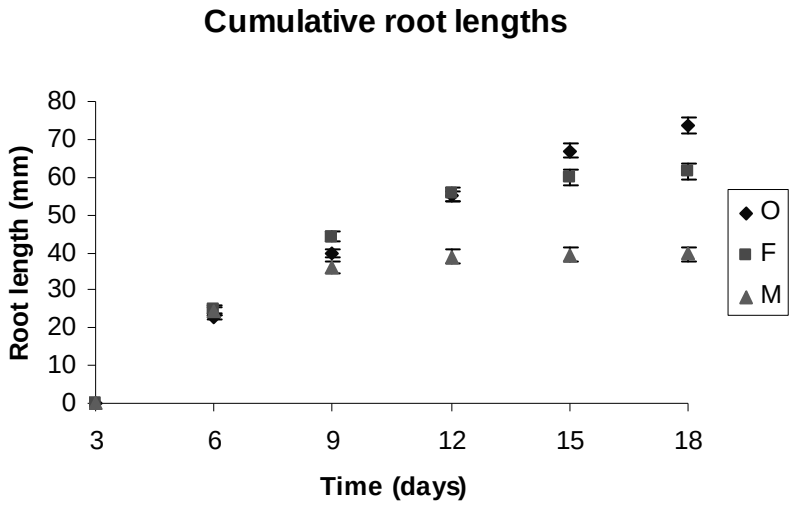

Figure 7.3 Cumulative length of primary roots of plants grown on agar plates without herbicide (O), with flumetsulam in the media (F) and with metsulfuron methyl in the media (M). Bars indicate the standard error of the mean.

Root tips of herbicide treated plants were distorted, becoming bulbous particularly in metsulfuron methyl treated roots, with the eventual loss of their root caps (Figure 7.2). Staining of primary roots revealed that the meristem and root cap cells were missing from herbicide treated plants. The dark staining vasculature on herbicide treated roots ended abruptly, in contrast to control plants where vasculature ended in a zone of dark staining meristematic region proximal to an intact root cap (Figure 7.4). This observation confirmed that the group B herbicides can terminate meristems.

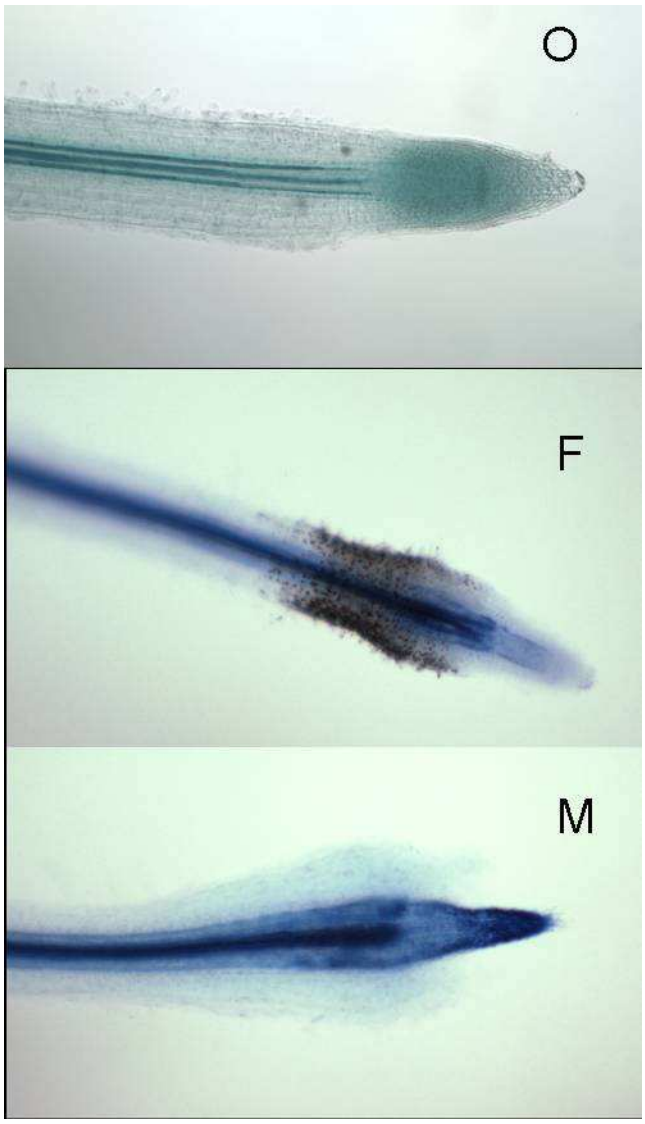

Figure 7.4 Methylene blue stained root tips of *Medicago truncatula* A17 grown on agar plates with no herbicide (O), flumetsulam (F) and metsulfuron methyl (M). A functioning meristem is evident in the control root, with a concentration of small undifferentiated cells. No meristem is evident in the herbicide treated roots, and the vascular bundle extends to the root tip. Loss of the root cap can also be seen. Dark staining structures in the epidermal cells of the flumetsulam treated root are evident.

Lateral root formation varied with herbicide treatment (Figure 7.7). Flumetsulam treated roots grew numerous short lateral roots. Plants grown on control plates had fewer ($p<0.001$) but longer lateral roots than the flumetsulam treated plants, whilst most metsulfuron methyl treated plants had negligible visible lateral roots (Figure 7.6).

Although metsulfuron methyl treated plants appeared to have no lateral roots, on closer inspection, small bumps on the primary roots of metsulfuron methyl treated plants were noted. Darker staining areas of vasculature and divided cells were observed after staining with methylene blue. Thus these bumps were identified as lateral root primordia (Figure 7.5). These primordia were not enumerated, so the frequency of the lateral root primordia expressed on a primary root length basis could not be calculated and compared to the frequency of visible lateral roots on control and flumetsulam treated plants. However it was evident that flumetsulam treated roots had a greater number of lateral roots than control plants, as might be expected where apical dominance is lost.

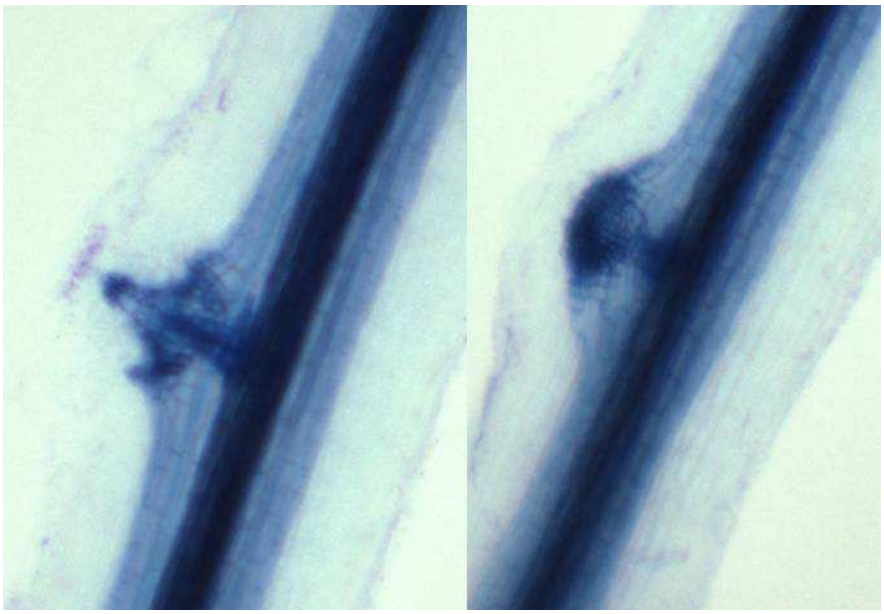

Figure 7.5 Lateral root primordia on metsulfuron methyl treated *Medicago truncatula* A17 roots.

Figure 7.6 *Medicago truncatula* A17 grown on agar plates for 18 days with no herbicide (O), flumetsulam (O) and metsulfuron methyl (M). Note the differences in primary root lengths, and the number and length of lateral roots. Plates are 15cm in diameter.

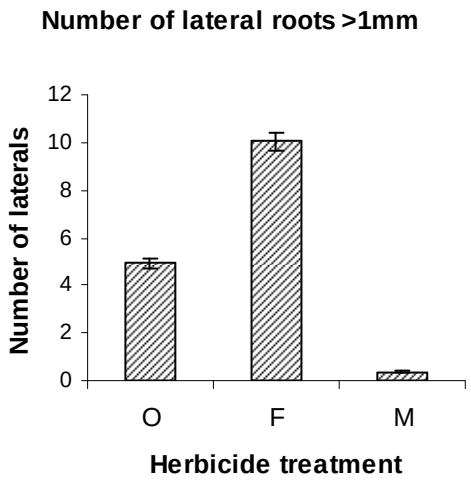

Figure 7.7 The number of visible lateral roots (>1 mm) of 18 day old plants grown on agar plates without herbicide (O), with flumetsulam in the media (F) and with metsulfuron methyl in the media (M). Bars indicate the standard error of the mean.

7.3.2 Proteome

The proteomes of 5 day old treated roots were mostly similar, with 53 protein spots detected as being differentially expressed between herbicide treated and untreated roots. Of these, 11 were identified by mass spectrometry (Table 7.1). A stress response was evident in herbicide treated plants, with the induction and accumulation of stress related small proteins, and reduced accumulation of metabolic proteins. In particular the PR10 protein, and the PR10 class and related proteins were induced, along with proteins involved in stress mediation including L-ascorbate peroxidase and 2,4-D-inducible glutathione S transferase. Enzymes involved in primary metabolism (phosphoglycerate mutase, enolase and glutamine synthetase) were less abundant in herbicide treated roots. However lactoylglutathione lyase accumulated in metsulfuron methyl treated roots, and the RuBisCO subunit binding protein alpha subunit accumulated in both herbicide treatments. Although both herbicide treatments elicited a similar pattern of changes to the proteome of root tips, notable exceptions were the increased accumulation of lactoylglutathione lyase and L-ascorbate peroxidise in metsulfuron methyl treated roots, and 2-4-D inducible glutathione S-transferase in flumetsulam treated roots. 2,3-bisphosphoglycerate independent phosphoglycerate mutase and glycine rich RNA binding protein 7 were down regulated in metsulfuron methyl treated roots but not in flumetsulam treated roots (Table 7.1).

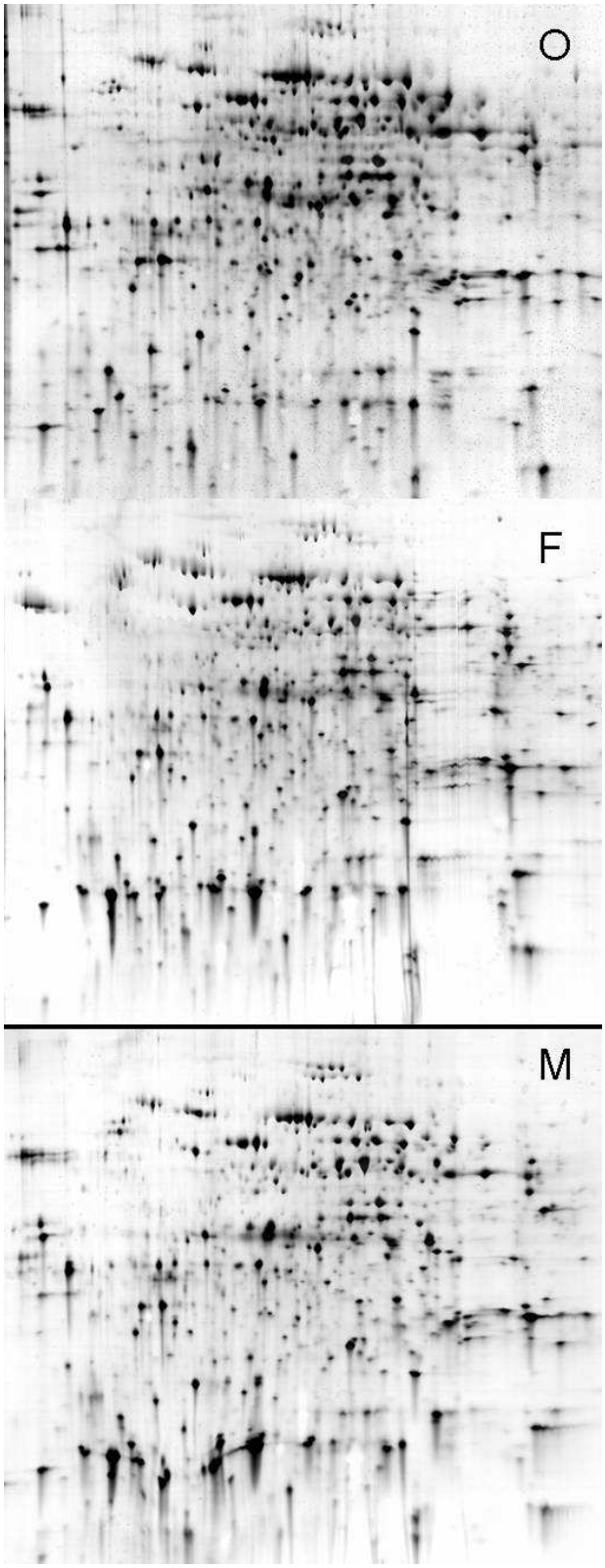

Figure 7.8 2-dimensional gels of proteins extracted from *Medicago truncatula* A17 grown on agar plates with no herbicide (O), flumetsulam (F) and metsulfuron methyl (M). The first dimension (horizontal) separates proteins based on their isoelectric point (4-7). Separation on the second dimension (vertical) is based in protein size.

Spot ID	%Vol for F[a]	%Vol for M[a]	%Vol for control[a]	P value[b]	LSD[c]	Fold change in %Vol for F[d]	Fold change in %Vol for M[d]	pI/M_r gel[e]	TIGR accession[f]	Best matching gene product[g]	MOWSE[h]	Matched peptides[i]	Sequence Coverage[j]	pI/M_r of protein match[k]	Type of MS
Primary metabolism															
40	0.78	0.083	0.784	<0.001	0.341	0.99	0.1	5.4/59.9	TC100321	2,3-bisphosphoglycerate independent phosphoglycerate mutase	66	4	6	9/92.8	TOF TOF
419	0.238	0.223	1.481	<0.001	0.503	0.16	0.15	5.7/43.3	TC100309	Enolase	116	9	31	6.1/61.9	MALDI TOF
164	0.286	0.221	0.524	<0.001	0.112	0.55	0.42	5.46/36.3	TC106729	Glutamine synthetase	103	2	3	7.4/57.7	TOF TOF
259	0.071	0.518	0.099	<0.001	0.15	0.7	5.24	5.2/24.1	TC95397	Lactoylglutathione lyase	83	2	8	8.3/36	TOF TOF
51	0.146	0.244	0.042	<0.001	0.107	3.5	5.9	4.4/57	TC100730	RuBisCO subunit binding-protein alpha subunit	187	6	12	5.3/70.1	TOF TOF
Defence and stress															
254	0.518	0.12	0.086	0.037	0.432	6	1.4	6.4/24.3	TC94259	2,4-D inducible glutathione S-transferase	72	19	6	8.3/44.9	MALDI TOF
268	0.046	0.5	0.077	<0.001	0.174	0.6	6.5	4.7/23.3	TC106425	L-ascorbate peroxidase	148	3	9	8/54.8	TOF TOF
287	0.192	0.06	0.152	0.03	0.102	1.2	0.39	4.9/17.6	TC96436	Glycine-rich RNA binding protein 7	135	3	39	4.8/16.3	TOF TOF
282	0.692	0.576	0.167	<0.001	0.902	4.1	3.4	4.9/15.2	TC67860	Pprg2 protein	111	3	21	5.8/16.6	MALDI TOF
295	0.231	0.183	0.048	<0.001	0.082	4.9	3.9	4.45/16.4	TC106613	PR10-1 protein	88	7	72	4.6/16.7	TOF TOF
311	0.343	0.33	0.404	0.037	0.619	0.85	0.82	3.9/15	TC106613	Class 10 PR protein	68	10	22	5.8/36.4	LC MS
Hypothetical															
246	0.126	0.064	0.501	<0.001	0.175	0.25	0.13	5.1/25.6	TC100694	ARD-like protein	72	5	2	9/57.7	TOF TOF
279	0.222	0.259	0.519	<0.001	0.123	0.436	0.5	4.4/18.6	TC106548	Early tobacco anther 1	91	5	13	5.1/34883	LC MS

Table 7.1 Proteomics results. [a)] % volume for each of flumetsulam (F) metsulfuron methyl (M) and control (C) treatments. [b)] P value and [c)] least squares difference (LSD) for REML analysis used to calculate significant changes in accumulation. [d)] Fold change in protein accumulation for treatment flumetsulam and metsulfuron methyl compared to untreated control roots. Isoelectric point (pI) and molecular weight (M_r) from the gel. [f)] Best matching tentative consensus (TC) sequence in the MtGI. [g)] Best matching gene product based on Mascot Blastx search. [h)] MOWSE score, scores over 66 are significant. [i)] Number of peptides matched to the translated TC sequence. [j)] Sequence coverage of matched peptides to the translated TC sequence. [k)] Predicted isoelectric point and molecular weight of the protein.

7.4 Discussion

By growing the model legume *Medicago truncatula* on agar plates containing the herbicides flumetsulam and metsulfuron methyl, differences in the early response of root morphology to these herbicides were identified. Proteomic analysis of root samples identified some differentially expressed proteins that aid the understanding of how these herbicides affect plants, with implications for symbiotic nitrogen fixation.

7.4.1 Root growth and morphology

Although a direct link between the experimental conditions in this work and what might be expected in the field cannot be assumed, the observed changes to root hairs and root development are likely to have implications for infection of root hairs by rhizobia and subsequent development of nodules. Few publications have described the effects of group B herbicides on legume roots and the implications of those effects for symbiotic nitrogen fixation. Martensson and Nilsson (1989) observed lucerne root hair deformations caused by chlorsulfuron (at approximately 4 g ha^{-1}) that were expected to inhibit the normal root hair infection process. The observed disruption to the apical meristems, with subsequent stimulation of lateral root primordia and then the inhibition of lateral root growth, may be indicative of effects group B herbicides have on nodule development.

The increase in lateral roots with flumetsulam was similar to observations in the vetch screening experiment (Chapter 3) where flumetsulam increased the number of non-viable 'pseudonodules' and points to a systemic effect on meristems. It was also consistent with observations that nodule mass rather than nodule number is inhibited by herbicide (e.g. the chickpea / imazethapyr experiment in Chapter 3) and supports the hypothesis that nodule development rather than initiation is inhibited. In addition, the post-sowing pre-emergent application of imazamox to pea plants reduced the observed number of nodules, while post emergent application increased the number of observed nodules. The apparently disparate results may just be different manifestations of the inhibition of meristems.

Common developmental pathways exist for lateral roots and nodules, as demonstrated by Bright *et al*. (2005). Similar changes to root hair length and density, abnormal root tip morphology, absence of root cap cells and loss of the apical meristem were common to herbicide treated (this chapter) and mutant *LATD* roots (Bright *et al*., 2005). Lateral root primordia were observed in metsulfuron methyl treated and *LATD* roots, but lack of subsequent meristematic development on lateral roots meant that they developed little beyond the primary root surface in both cases. Nodule development in *LATD* mutants was clearly defective. The small white nodules were consistent with a lack of full differentiation, and no nitrogenase activity. Such small white nodules were observed in the screening work described previously (e.g. vetch in Chapter 3). Infection by rhizobia and bacteroid development was impeded in the *LATD* mutant, suggesting that bacteroid development is closely linked to plant root development. A similar disruption to plant root development and hence nodulation and nitrogen fixation might be expected in herbicide treated roots.

7.4.2 Differentially expressed proteins

The identified differentially expressed proteins can be classed into primary metabolism proteins and defence / stress proteins. The primary metabolism proteins were down regulated, except for lactoylglutathione lyase (glyoxylase). Glyoxylase is involved in metabolising methylglyoxyl, a metabolite that inhibits the growth of cells. Glyoxylase is therefore thought to have a role in tolerance to stress (Veena *et al*., 1999). Glyoxylase I activity is increased in rapidly dividing and non-differentiated cells, and increased activity has also been reported for treatments that stimulate growth, such as hormones and light. Although glyoxylase I has been used as a ‘marker’ for growth, its up-regulation has also been reported in response to salt stress (Espartero *et al*., 1995). The 5-fold increased accumulation of glyoxylase in metsulfuron methyl treated roots at the time of sampling is well reconciled by the stress observed in the roots at later time-points, but could also be indicative of a higher proportion of non-differentiated cells.

Glutamine synthetase (GS) is the main point of regulation of nitrogen metabolism, catalysing the assimilation of inorganic ammonium to an organic form of nitrogen – namely glutamine (see reaction equation below). Glutamine synthetase from

leguminous plants has been well studied because of its role in assimilation of ammonium from nitrogen fixation.

$$\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + P_i + H^+$$

There are a number of GS isozymes in plants. Cytosolic GS is particularly important for nodules (Stanford *et al*., 1993). The relative abundance of GS in roots of herbicide treated plants was approximately half that of control plants. This is in agreement with Scarponi *et al.* (1997) where *Vicia* faba treated with chlorimuron-ethyl or imazethapyr had 60% less GS activity than control plants. Although the reductions in nitrogen assimilation can be interpreted to be a result of reduced growth and hence nitrogen demand of the plants, it appears that it is more intimately linked with reduced branched chain amino acid (BCAA) synthesis caused by the inhibition of AHAS. Scarponi *et al*. (1997) showed that the inhibition of AHAS activity reduced the free BCAA pool, but caused the accumulation of other amino acids. This was most likely due to the degradation of pre-existing proteins, resulting in no significant net change to the total amino acid pool size. A decrease in protein concentration in the shoots resulted from a disturbance to free amino acid levels, and there was a decrease in total nitrogen, but an increase in soluble nitrogen. The abrupt decline in AHAS activity after treatment was accompanied by declines in glutamate synthase and then GS. Thus by disrupting protein synthesis, it appears that group B herbicides actually reduce the demand for nitrogen and hence nitrogen assimilation. This is expected to have direct consequences for nitrogen fixation.

Along with the implicit reduction in nitrogen assimilation caused by the group B herbicides, the proteomics results indicate that energy production by the glycolytic pathway is also disrupted. Enolase catalyses the penultimate step in glycolysis by forming phosphoenolpyruvate from 2-phosphoglycerate. Enolase was less abundant in both herbicide treatments compared to the controls. Enolase catalyses the only dehydration step in the glycolytic pathway (see Figure 7.9). 2,3-bisphosphoglycerate independent phosphoglycerate mutase catalyses the step before enolase in the glycolytic pathway. The flumetsulam treatment did not alter the relative abundance of phosphoglycerate mutase compared to control plants, but only one tenth of the relative abundance of this enzyme in control plants was found in metsulfuron methyl treated

roots. The distinct difference in accumulation of phosphoglycerate mutase between metsulfuron methyl and flumetsulam treatments can be reconciled with the relative potency of these herbicides, and a possible regulatory cascade up the glycolysis pathway. Thus as the plant response to herbicide progresses, pyruvate would accumulate due to inhibition of AHAS (Gaston *et al*., 2002). In this experiment the metsulfuron methyl treated roots were at a more advanced state of inhibition than flumetsulam treated roots. The proposed chain of inhibition starting with AHAS, leading to increased pyruvate availability, then inhibition of enolase followed by the inhibition of phosphoglycerate mutase is consistent with the proteomics results. Furthermore, Co-enzyme A is produced from pantoate off the BCAA synthesis pathway and is required for pyruvate to enter the TCA cycle as acetyl CoA. Pyruvate is a central molecule in cellular metabolism. Thus the inhibition of AHAS is expected to have profound effects on cells.

Figure 7.9 The glycolytic pathway.
http://biotech.icmb.utexas.edu/glycolysis/pathway.html

Ribulose 1,5-bisphosphate carboxylase (RuBisCO) is the limiting step of carbon fixation. The RuBisCO binding protein (RBP or cpn60) is a 60 kDa chaperonin essential for correct protein folding of RuBisCO in stressed and unstressed cells (Musgrove *et al*., 1987). RBP is suggested to function as a heat shock protein (Demirevska-Kepova *et al*., 2005) that plays a protective role under high temperatures.

The increased levels of this chaperonin in herbicide treated roots suggests a wider role for RBP in the stress response, since carbon fixation by RuBisCo in roots does not occur.

A number of stress response proteins were up-regulated in herbicide treated root tips, including PPRG2 which is expressed in response to pathogens such as the parasitic plant *Custer* spp. (Borsics and Lados, 2002) and PR 10 proteins which have been studied in *Medicago sativa* (Breda *et al*., 1996). Similarly Scarponi *et al.* found that soybean plants treated with imazethapyr had increased abundance of key enzymes for phytoalexin synthesis which are produced as a natural response to biotic and abiotic infections (Scarponi *et al*., 1996). The question remains as to why a chemical induces a pathogenesis related protein. The experiments were conducted aseptically on agar plates, making the presence of pathogens unlikely. The integrity of the root epidermis may be compromised in herbicide treated roots, and may be a common means by which a pathogen related stress response is stimulated. Although the PR 10 class proteins are known to be up-regulated in response to pathogens, it may be the case that they can form part of a general defence reaction to abiotic stresses.

L-ascorbate peroxidase is a major enzyme involved in responses to oxidative stress. Flumetsulam did not induce a significant change in the abundance of this protein. However there was a 6 fold increase in this enzyme in metsulfuron methyl treated plants. Zabalza *et al*. (2007) argue that oxidative stress is not directly related to the mode of action of AHAS inhibiting herbicides. The authors did not observe an increase in ascorbate peroxidase in the roots of pea plants treated with imazethapyr for up to 15 days. Thus the increased expression of ascorbate peroxidase in metsulfuron methyl treated plants may be indicative of the advanced stage of herbicide injury to the plant and/or the higher potency of metsulfuron methyl compared to other group B herbicides.

Glutathione S-transferase (GST) proteins are directly involved in detoxifying herbicides and other xenobiotic compounds (Marrs, 1996). They catalyse the conjugation of glutathione to hydrophobic, electrophilic substrates. GSTs conjugate natural products such as anthocyanins, can act as binding proteins for plant hormones and catalyse glutathione-dependent peroxidase and isomerase reactions (Edwards, 1996; Marrs, 1996) although fulfilling roles in the normal functioning of cells, GSTs have mostly

been studied for their ability to metabolise herbicides to non-toxic intermediates. GSTs have a direct role in the tolerance of plants to herbicides. Safeners work by inducing GSTs in plants so that herbicides can be more effectively detoxified. The very large increase in the abundance of a GST in flumetsulam treated roots but not in metsulfuron methyl treated roots may be directly responsible for the greater tolerance of A17 to flumetsulam.

RNA binding proteins are involved in processing RNA. It is suggested that glycine rich RNA binding proteins are involved in stress response (Nomata *et al*., 2004), but their physiological function remains largely unknown. It is therefore not possible to comment on the significance of the decrease in glycine rich RNA binding protein 7 in metsulfuron methyl treated roots, other than its coincidence with more severe symptoms for this herbicide compared with flumetsulam.

7.4.3 Implications

While no attempt has been made to relate the root observations and proteomic results to what might occur in the field, the results of this work are of interest because they can direct future studies into the mechanisms by which group B herbicides reduce nitrogen fixation. Symptoms were initially observed in the root hairs. Disrupted root hair infection by rhizobia is a likely cause of reduced nodulation, as raised by Martensson and Nilsson (1989). The screening experiments, particularly the chickpea / imazethapyr experiment, highlighted impeded root growth as a likely factor in reduced nitrogen fixation, especially where the herbicide resides in the soil. The screening experiments were limited to measurements of root dry weight, nodule number and nodule dry weight. The expression of nodule data on a root mass basis gave some idea as to whether nodule mass (indicating nodule development) or nodule number (indicating initiation of nodules) was more sensitive to herbicide. In some of the screening experiments (for example chickpea / imazethapyr), nodule dry weights were reduced, but nodule numbers either absolute or on a root mass basis were not affected to the same extent. However the screening experiments could not be conclusive because of the timing of herbicide application in relation to the development of nodules. Where nodule initiation had occurred prior to herbicide application, a reduction in nodule number might not be observed even if the herbicide can inhibit nodule initiation. In the

current agar experiment, the initiation of lateral roots was not affected even at the high concentrations of herbicide. The increase in lateral root numbers with flumetsulam and the presence of lateral root primordia in metsulfuron methyl treated roots indicates that at a plant cellular level nodule development rather than nodule initiation is likely to be inhibited. Of course, this does not take into account the effects that root hair deformation would have on infections and nodule initiation. In the screening experiments increased numbers of small, ineffective nodules were often observed (for example vetch / flumetsulam) and are likened to the small bumps observed on metsulfuron methyl treated roots in this chapter.

The identified differentially expressed proteins mostly relate to a stress response. It is well established that nitrogen fixation is down-regulated in response to stress. This pathway of reduced nitrogen fixation is likely to be elicited by group B herbicides. The glutathione S transferase identified is a direct response to a xenobiotic compound, and may be a useful marker of herbicide tolerance for future studies. It might also find use in a bioassay to detect low levels of herbicide residing in soil.

The decreased prevalence of glutamine synthetase is of greater direct relevance to nitrogen fixation. The physiological reason for the down-regulation of this protein is likely to be the reduced demand for nitrogenous compounds in plants in which growth is impeded. It is also plausible that the perturbation in free amino acid concentration signals an oversupply of nitrogenous compounds and leads to the down-regulation of nitrogen assimilation. The reduced abundance of GS in this experiment provides evidence for plant mediated control of nitrogen demand, and hence nitrogen fixation in response to group B herbicides. The reduction in glycolysis proteins also indicates a close link between AHAS inhibition and primary cellular metabolism.

7.4.4 General experimental considerations

These experiments were aimed at identifying the effects of group B herbicides on early plant development, particularly changes in the proteome of root tips. As such, they were not designed to induce symptoms to a degree that would be expected in the field, but rather to indicate what the symptoms are, the underlying cellular processes, and the

possible implications for nitrogen fixation. The growth of medicago in this work was on agar plates for ease of observation, harvesting of roots and uniform treatment effects. Furthermore, high rates of herbicide were used, and no attempt to link concentrations of herbicide in agar to a spray rate onto soil has been made.

The harvest for proteomic analysis was limited to one time. Earlier harvest times are more likely to detect the initial effects of herbicide. Harvesting at later time points are more likely to detect changes, but may be a secondary response to the initial changes, or indeed produce the proteome of a near dead plant. The time point for harvest, at 5 days, was sufficient for adequate growth to have occurred in the plant. At this time, visible symptoms were only just noticeable, but no necrosis was evident.

In proteomics work there are restrictions on what proteins are identifiable, inherent upon the methodology used. The gels used in this work can only distinguish proteins with an isoelectric point between 4 and 7. Larger and more abundant proteins are more likely to be detected than less abundant proteins, such as the small regulatory proteins. This is in part determined by the sensitivity of the gel staining, and the ability to accurately quantify the protein abundance using the gel analysis software. Finally, the ability to identify differentially expressed proteins relies on past work and identification by mass spectrometry. Although the proteins identified here will only be a subset of all proteins that are differentially expressed, they do provide some useful insights into the response of the plant to these herbicides.

7.4.5 Conclusion

By growing model legume *Medicago truncatula* on agar plates containing herbicide, making morphological observations on the developing roots, and conducting a proteomic analysis of root tips, some valuable insights into the mechanisms by which group B herbicides might affect nitrogen fixation were obtained. Flumetsulam and metsulfuron methyl reduced root hair length and density in agreement with previous work. These morphological changes are likely to inhibit the infection of roots by rhizobia to some degree. As the effect of group B herbicides on root morphology progresses, it becomes evident in the meristems. Primary root elongation was impeded, and root tips later became bulbous with the concomitant loss of the root cap cells. With

the primary root becoming determinate, an increase in the number of lateral roots occurred in flumetsulam treated plants. At later stages, the lateral root meristems underwent the same fate as the primary root meristems. The metsulfuron methyl treated plants also provided evidence that meristem development rather than initiation was affected by group B herbicides. A number of lateral root primordia were observed, but these did not develop beyond a bump on the primary root. Thus, the observations of root morphology are consistent with previous publications and the implications for nodulation in line with observations in the previous chapters.

The identified differentially expressed proteins consist mainly of primary metabolism proteins that were less abundant, and stress response proteins that were more abundant in the herbicide treated plants. 2,4-D inducible glutathione S-transferase was 6 fold more abundant in flumetsulam treated plants, but only increased by 40% in metsulfuron methyl treated plants. Whether this protein is responsible for the less severe effects of flumetsulam compared to metsulfuron methyl is of interest, and it may be a useful marker for tolerance to particular herbicides. Of the glycolysis proteins identified, 2,3-bisphosphoglycerate independent phosphoglycerate mutase was down-regulated in metsulfuron methyl treated plants, and enolase was down-regulated in both flumetsulam and metsulfuron methyl treated plants. With metsulfuron methyl being the more severe of the two herbicides, the different responses suggest a stepped regulation cascade whereby enolase is first inhibited, perhaps in response to increased levels of pyruvate. Phosphoglycerate mutase is subsequently inhibited. Of direct relevance to nitrogen metabolism and hence nitrogen fixation, glutamine synthetase was less abundant in herbicide treated roots, indicating that less nitrogen assimilation was occurring. Whether this is in response to decreased nitrogen demand due to less plant growth, or is more intimately connected with the perturbation to plant biochemistry should be explored in the future.

Chapter 8 General discussion

This thesis examines the impact of group B (acetohydroxyacid synthase inhibiting) herbicides on nitrogen (N_2) fixation by the legume / rhizobia symbiosis formed by many of the major grain and pasture legumes grown in southern Australia. Prior to this work, evidence from a limited number of legume species and herbicides indicated that N_2 fixation was inhibited (see Chapter 2). Most of the publications that looked more directly at which symbiont was affected by herbicide indicated that the plant and not the rhizobia was the susceptible symbiont. This was based on rhizobia growth only being affected at very high herbicide concentrations during *in vitro* experiments (see Chapter 2) and is consistent with the incubations in Chapters 3, 4 and 7. However previous work (Anderson *et al*., 2004) indicated that the rhizobia were also susceptible since exposure to herbicide prior to inoculation reduced subsequent nodulation and nitrogen fixation of chickpea.

The first aim of this work was to determine the extent to which recommended group B herbicides applied in-crop have the potential to reduce nitrogen fixation in pasture and grain legumes commonly grown in southern Australia. The second aim was to investigate the mechanisms by which group B herbicides inhibit nitrogen fixation. Clarification was sought on whether the rhizobia were susceptible to the herbicide or the impacts on nitrogen fixation were purely via an effect on the legume. The identification

of biochemical and morphological influences of group B herbicides on the legume provided further exploration of the mechanisms.

In this final discussion, the results from the preceding chapters are summarised and the implications are placed in context of the current literature. Remaining questions and possible future work are identified.

8.1 Summary of experimental work

8.1.1 Screening experiments:

The screening experiments assessed the effect of three recommended in-crop group B herbicides (flumetsulam, imazethapyr and imazamox) on the biomass, nitrogen content and nodulation of four grain legumes (chickpea, pea, faba bean and vetch) and four pasture legumes (lucerne, burr medic, subterranean clover, balansa clover). It was confirmed that in some instances in-crop group B herbicides reduce nitrogen fixation. The impacts of herbicide treatment ranged from no effect to severe restriction of growth and nodulation (mass and number). The experiments were designed with factorial treatments; with application of herbicide to the plant as per label recommendations, as well as the exposure of rhizobia to herbicide prior to inoculation. While there were often clear effects of the herbicide on the plant, the effects on rhizobia and interactions between the two treatments were more difficult to explain. The variation in responses of the legume by herbicide combinations imply that there is an opportunity to be selective in weed control decisions to avoid disruptions to nitrogen fixation. However in the field, responses are likely to be influenced by soil and climatic conditions.

In order to be more certain of the contribution of symbiotically fixed nitrogen to farming systems where group B herbicides are used, a better understanding of the mechanisms by which N_2 fixation can be inhibited by these herbicides was required.

8.1.2 Herbicide tolerant *Medicago littoralis* FEH-1:

By comparing a herbicide tolerant *Medicago littoralis* cultivar (FEH-1) to its parent, Herald, it was demonstrated that the reduction in N_2 fixation caused by herbicide was mainly determined by the tolerance of the plant to the herbicide. The application of

chlorsulfuron equivalent to 0.75 g ha^{-1} reduced the biomass and nitrogen content of shoots and roots to a greater degree in Herald plants than in FEH-1 plants. Similar results were found for nodule dry weight and number, and acetylene reduction assays showed that the activity of the nitrogenase enzyme was lower in herbicide treated Herald plants than FEH-1 plants. It was expected that the tolerance of FEH-1 to herbicide would completely remove plant effects of the herbicide and clearly indicate any direct effects of the group B herbicides on rhizobia. However it was found that FEH-1 was not completely tolerant to the herbicide. Thus while a relationship between the tolerance of the plant to herbicide and growth, nodulation and nitrogen fixation could be demonstrated, this experiment could not conclusively eliminate some contribution of rhizobia to the susceptibility of the symbiosis to herbicide.

8.1.3 Proteomics and root morphology:

By examining the roots of *Medicago truncatula* A17 grown on agar plates in the presence and absence of metsulfuron methyl and flumetsulam and conducting a proteomic analysis of the root tips, some morphological and biochemical effects in the plant that have the potential to interfere with nitrogen fixation were identified. Abnormal root hairs and the disruption of root meristems were observed and if replicated in field conditions would be likely to inhibit nodule initiation and development. Changes to key metabolic pathways and stress responses were evident in the proteomes of roots exposed to herbicide. The abundance of glutamine synthetase in herbicide treated roots was half that of control roots, indicating a reduced ability to assimilate nitrogen which has direct implications for nitrogen fixation. Glycolysis enzymes were also less abundant in herbicide treated roots and can be traced through biochemical pathways to the inhibition of the acetohydroxyacid synthase enzyme by herbicide. The disruption of this primary metabolic pathway could result in a myriad of secondary effects. The expression of a glutathione S-transferase (GST) in flumetsulam treated roots and absence in metsulfuron methyl treated roots corresponded with a less severe retardation of growth and other symptoms in the flumetsulam treated roots. GSTs are involved in the detoxification of xenobiotic compounds and may be indicative of a more successful defence response in flumetsulam treated roots compared to metsulfuron methyl treated roots.

8.2 Mechanisms

The mechanisms by which group B herbicides reduce nitrogen fixation can be informed by two major areas of research that are the subject of ongoing investigation:

1) There is currently no unifying theory on how nitrogen fixation in legumes is regulated at the whole plant level. Three plausible theories were discussed by Schulze (2004); i) the regulation of carbohydrate supply; ii) the regulation of oxygen diffusion into the nodules; and iii) N feedback. Herbicides may inhibit nitrogen fixation by invoking any of these regulatory mechanisms, but more direct effects are possible if there is a more specific link between the inhibition of the AHAS enzyme and nitrogen fixation.

2) While the primary mode of action of group B herbicides has been defined, it is still not known how this mode of action results in mortality of intolerant plants (Gaston *et al*., 2002; Moberg and Cross, 1990; Royuela *et al*., 2000). If the specific mechanisms of plant death can be delineated, the potential implications for nitrogen fixation by legumes chronically affected by the same herbicides can be deduced.

Although the exact mechanism of lethality of group B herbicides is unknown, many biochemical and morphological consequences have been described. Each biochemical or morphological perturbation could have different implications for nitrogen fixation depending on the severity of the response to herbicide and the stage at which herbicide is applied.

The biochemical consequences of group B herbicides on plants can be envisaged as a cascade of 'effects' (see Figure 8.1). The primary effect of group B herbicides is to inhibit the acetohydroxyacid synthase enzyme. Due to the lack of new branched chain amino acids, 'de novo' synthesis of protein ceases (Zabalza *et al*., 2006), although protein turnover allows the plant to scavenge some amino acids in the short term. Both an increase (Gaston *et al*., 2002; Royuela *et al*., 2000) and a decrease in free amino acids (Scarponi *et al*., 1997) have been reported in the literature, however in either case there is likely to be an undersupply of branched chain amino acids compared to the other amino acids. The increase in soluble N (Scarponi *et al*., 1997), consistent with increased levels of free amino acids might provide a signal that there is a sufficient

supply of nitrogen to the plant. The regulation of N_2 fixation via an N feedback mechanism involving free amino acids has been suggested elsewhere (Hartwig and Trommler, 2001). Thus by inhibiting the production of branched chain amino acids and simultaneously causing a perturbation in free amino acids, it can be hypothesised that the N feedback regulation of N_2 fixation may be indirectly switched on by group B herbicides. This is consistent with the reduced abundance of glutamine synthetase in herbicide treated roots in the proteomics work (Chapter 7), as well as the decrease in nitrate uptake in roots of soybean plants treated with imazethapyr and the concomitant reduction in translocation of N to the shoots and the inhibition of nitrate reductase activity in shoots and roots (Zabalza *et al*., 2006). If group B herbicides affect the assimilation of nitrate from the soil in this way, it can be assumed the inhibition of nitrogen fixation will occur in symbiotic plants.

The cessation of growth of plants and hence sink strength for nitrogen is an even more indirect route for invoking N feedback regulation. In the chickpea / imazethapyr and pea / imazamox experiments (Chapter 3) and when flumetsulam was applied to balansa clover and subterranean clover (Chapter 4) the nitrogen concentrations of the plant tissues was not affected by the herbicide. Thus the amount of nitrogen fixed was strongly coupled to plant growth. In other experiments an increase in nitrogen concentration was observed (balansa clover / imazamox, lucerne / flumetsulam, vetch / flumetsulam, lucerne / imazamox in Chapter 4 and for FEH-1 shoots in Chapter 6) indicating a more severe effect of herbicide on growth than on nitrogen fixation. Under the experimental conditions imposed in this thesis there was little evidence for reductions in nitrogen concentration of plant tissues and accordingly it was unlikely that nitrogen fixation was affected independent of effects on plant growth. However it is still possible that under field conditions nitrogen fixation could be more susceptible than plant growth. The cessation of growth can result from insufficient protein synthesis caused by the inhibition of BCAA synthesis. Voisin *et al*. (2003) found that nodules in pea plants had the highest demand for carbon in the vegetative growth stage, growing at the expense of roots and shoots. Therefore the inhibition of growth might actually have a greater effect on nitrogen fixation through a more severe reduction in nodule development and photosynthate supply to nodules in particular, compared to the overall effect on plant growth and the amount of nitrogen fixed as a function of lower biomass.

Such a mechanism may have occurred in the chickpea / imazethapyr experiment in Chapter 3 where there was a reduction in nodule mass per unit root mass in response to post-sowing pre-emergence herbicide application. Other experiments where there was a reduction in nodule mass per unit root mass were the vetch / flumetsulam experiment in Chapter 3, subterranean clover / flumetsulam in Chapter 4 and for FEH-1 and Herald cultivars of *Medicago littoralis* in Chapter 6. However due to the timing of herbicide application in these experiments, this evidence was not conclusive. Plant growth might also cease due to the toxicity of chemicals that build up due to the inhibition of the AHAS enzyme (La Rossa *et al*., 1987). Accordingly chlorsulfuron caused ultrastructural changes in root and leaf cells consistent with toxicity from intermediates before changes consistent with amino acid starvation (Stoynova *et al*., 1997). The effects of toxic levels of certain chemicals and also resulting changes in plant physiology are therefore another possible mechanism for herbicide impacts on nitrogen fixation.

Observations of the effects of metsulfuron methyl and flumetsulam on roots of *Medicago truncatula* in Chapter 7 indicated that the morphological consequences of group B herbicides result from the perturbation of meristems. This initially manifested in deformed or sparse root hairs followed by impaired development of roots and nodules. With fewer and deformed root hairs, infection by rhizobia is likely to be impeded. This should result in a reduction in the number of nodules, and was observed for lucerne / imazamox and lucerne / imazethapyr experiments in Chapter 4. Another observation was the increase in the number of lateral roots in flumetsulam treated roots and lateral root primordial in metsulfuron methyl treated roots in Chapter 7. This indicated a loss of apical dominance, and is similar to the increase in nodule number in flumetsulam treated vetch plants and pea plants treated with post emergent imazamox in Chapter 3. However even if nodule initiation and infection is successful, further developmental problems can occur. Accordingly the increase in nodule number on flumetsulam treated vetch roots was due to a large number of small, white nodules that would not be fixing N_2. Thus both nodule initiation (due to deformed root hairs as in Chapter 7 and Martensson and Nilsson (1989)) and nodule development (due to inhibition of meristems as observed in Chapter 7) are likely to be impeded by group B herbicides. While reductions in nodule number and nodule mass were observed in Chapter 3, 4 and 6, the extent to which nodule initiation or nodule development is

inhibited will depend on the timing of herbicide application and the potency and dose of the herbicide.

Alternative metabolic pathways are stimulated by group B herbicides. This could be in response to the disruption of primary metabolic pathways as demonstrated in Chapter 7. Fermentative pathways (Gaston *et al*., 2002) and the alternative oxidase (Gaston *et al*., 2003) have been observed in pea and soybean plants in response to imazethapyr. This is consistent with findings of Frechilla *et al*. (2002) who observed alternative pathway respiration in pea roots supplied with excess ammonium. The increase in free amino acids due to excess ammonium supply (Frechilla *et al*., 2002) was common to observations after herbicide application (Gaston *et al*., 2002; Rhodes *et al*., 1987; Royuela *et al*., 2000), as was the decrease in glutamine synthetase activity observed in the proteomics work and by Scarponi *et al*. (1995). Frechilla *et al*. (2002) proposed that an increase in glutamate dehydrogenase could have a role in ammonium detoxification, together with alternative respiration pathways to provide C skeletons for ammonium assimilation whereas surplus NADH is oxidised via the non-phosphorylating route.

Zabalza *et al*. (2007) argue that oxidative stress is not directly related to the mode of action of AHAS inhibiting herbicides. They propose that the inability of herbicide treated plants to respond to oxidative stress relates to the modification of protein synthesis caused by the inhibition of AHAS. However the proteomic analysis of metsulfuron methyl treated roots (Chapter 7) showed a 6 fold increase in L-ascorbate peroxidase, an important enzyme involved in the response to oxidative stress.

Decreased photosynthesis might be observed after exposure of plants to group B herbicides but is likely to be a downstream response due to less demand for photosynthate or a failure of other biochemical processes essential to maintain photosynthesis (Cobb, 1992). The restriction of photosynthate supply to nodules due to group B herbicides might occur due to the inhibition of stomatal conductance and photosynthesis, as suggested for soybean plants (Zabalza *et al*., 2006). Accumulation of starch and sugars in pea plants in response to imazethapyr indicated an oversupply of carbohydrates due to a decrease in sink strength (Gaston *et al*., 2002). However Royuela *et al.* (2000) showed that long term imazethapyr treatment of pea plants did not

impair the capacity to synthesise carbohydrate, as there was a negligible effect on photosynthesis. No significant changes in carbohydrate levels were detected in nodules either, which suggests that sucrose availability for nodules was not limiting for nitrogen fixation (Royuela *et al*., 2000). Thus although group B herbicides might restrict photosynthesis, the initial effects of the herbicide will be on N metabolism and growth and immediate effects on N_2 fixation are more likely to be mediated in this way than on the restriction of photosynthate supply to the nodules.

Strong links between amino acids, plant N biochemistry and nitrogen fixation have recently been discovered. Thus there is possibly a more direct link between the inhibition of AHAS and levels of nitrogen fixation. By mutating the general ABC amino acid transporters BraDEFGC and AapJQMP in *Rhizobium leguminosarum*, it was demonstrated that amino acids are necessary for the transfer of nitrogen between bacteroids and pea plants (Lodwig *et al*., 2003). Further work has shown that when only BraC3 is expressed, wild type nitrogen fixation occurs. BraC3 is very specific for the aliphatic amino acids alanine, valine, leucine and isoleucine. Microarray data (Karunakaran *et al*., 2009) and quantitative RT-PCR shows that transcripts for the key enzymes of the biosynthetic pathways of the branched chain amino acids (e.g. ilvC, ilvE2, leuC and leuD) are severely down regulated in bacteroids (Prell *et al*., 2008), suggesting that any requirement for branched chain amino acids must be supplied by the plant. This is in contrast to Royuela *et al*. (1998) and Zawoznik and Tomaro (2005) who demonstrated high AHAS activity in bacteroids. Although it was hypothesized that rhizobia unable to produce branch chain amino acids will not be impeded in the symbiosis, an *IlvD* and a *LeuD* mutant were not able to nodulate pea plants. This is consistent with other findings (Aguilar and Grasso, 1991) where an *IlvC* mutant *Rhizobium meliloti* 1028 was unable to nodulate *Medicago sativa*. The *LeuD* mutant could nodulate with the addition of leucine to the nodulation medium and fix nitrogen at wild type levels. The authors concluded that symbiotic auxotrophy is not general, but specific to the branch chain amino acids and that wild type nitrogen fixation rates are dependent on the transport of these amino acids (Prell *et al*., 2008).

It is plausible that if BCAA synthesis in rhizobia is compromised by group B herbicides the symbiosis will be affected. This could provide some explanation for the treatment effects of rhizobia pre-exposure in this thesis and by Anderson *et al*. (2001; 2004).

Aguilar and Grasso (1991) demonstrated that a particular step in the symbiosis is affected by a mutation in the *ilvC* gene. Luteolin is an inducer molecule produced by the legume which induces *nod* gene expression in the rhizobia. Nod gene expression was not induced by luteolin in the IlvC$^-$ mutant. Thus there is a specific requirement for acetohydroxyacid isomeroreductase in the rhizobia for nodulation. It is not known whether specific products in the BCAA pathway are required or inhibit *nod* gene expression, or the isomeroreductase enzyme processes some other chemical that is required. Nodulation by the *LeuD* mutant described by Prell *et al*. (2008) was rescued by the addition of leucine and suggests that at least this branched chain amino acid is required for nodulation of peas.

More recently des las Nieves Peltzer *et al*. (2008) published a comprehensive assessment of nodulation by *Sinorhizobium meliloti* mutants in the branched chain amino acid synthesis pathway. In contrast to previous findings with *S. meliloti* (Aguilar and Grasso, 1991) all auxotrophic mutants for isoleucine and valine pathway (*ilvI, ilvC* and *ilvD2*) and for leucine (*leuA1, leuC, leuD* and *leuB*) in two strains of *S. meliloti* were severely deficient in their ability to nodulate and infect. Other studies (Hasani *et al*., 2002; Lopez *et al*., 2001; Sanjuan-Pinilla *et al*., 2002; Truchet *et al*., 1980) have also shown that BCAA auxotrophs are symbiotically compromised. des las Nieves Peltzer *et al*. (2008) concluded that BCCAs are essential for nodulation and infection. Since nodulation can be rescued by supplying the amino acid in the media, the actual phenotype will depend on the availability of amino acids in root exudates and in the plant. The authors suggest that in soil, the ability of wild-type rhizobia to synthesize amino acids that might be limiting in the rhizosphere of their host plants is essential for the establishment of an effective symbiosis and the competitiveness of the rhizobia.

Currently a synthesis of the observed effects of group B herbicides on N metabolism does not provide a cohesive sequence of events that might lead to the inhibition of nitrogen fixation. However the weight of evidence indicates that the metabolic disturbance caused is consistent with observed reductions in the amount of nitrogen fixed recorded in this thesis and by others. As it becomes established that branched chain amino acids are essential for symbiosis in a direct way, plausible mechanisms for the inhibition of nitrogen fixation by group B herbicides can be envisaged. Morphological effects on root hairs and meristematic development will have more

general effects on infection and nodule development. Taken together, a growing body of evidence suggests that if BCAA synthesis in either symbiont is compromised by group B herbicides, the formation and function of the symbiosis is likely to be affected. (Figure 8.1).

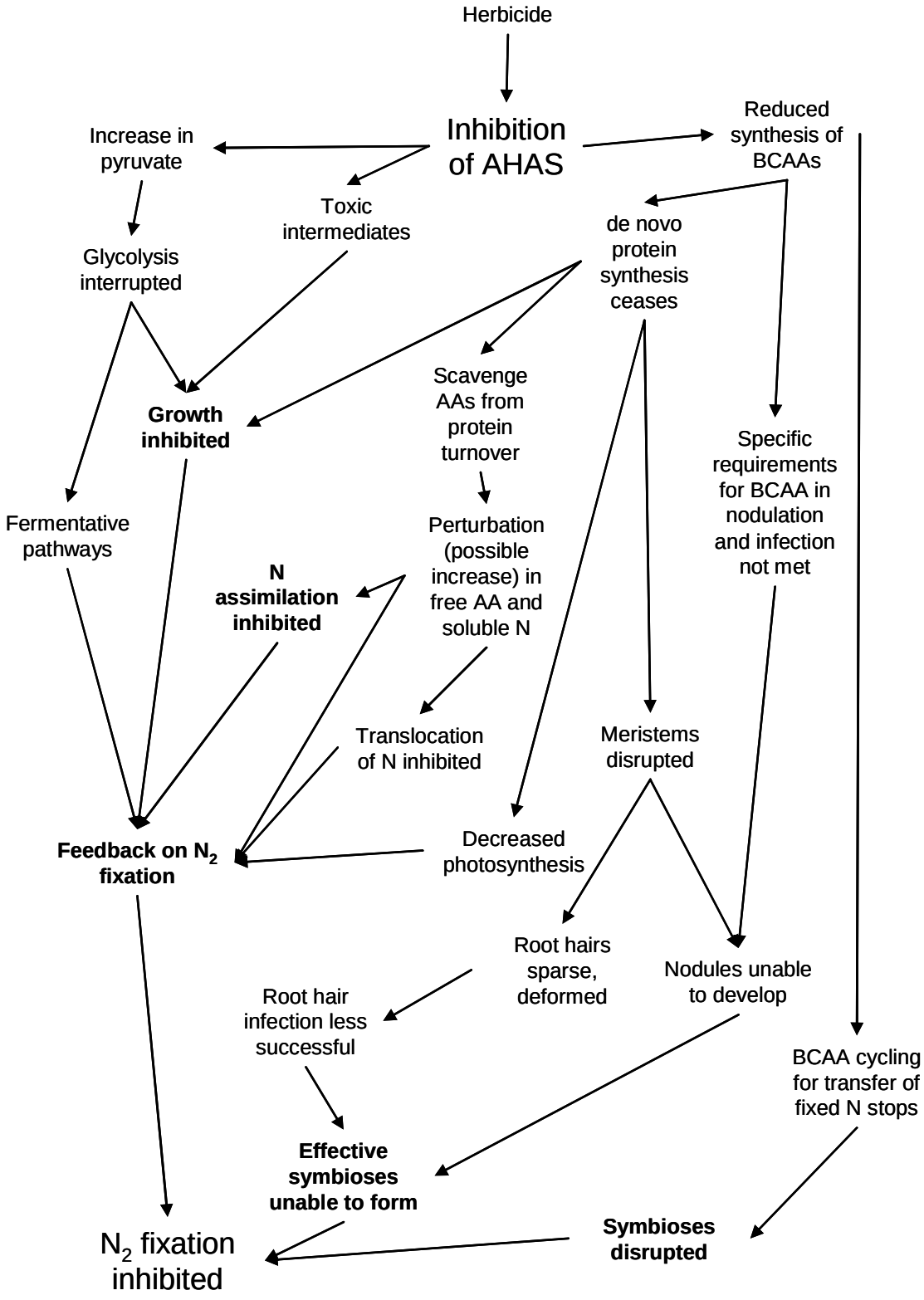

Figure 8.1 Potential mechanisms for the inhibition of nitrogen fixation by group B herbicides derived from experimental results and the literature.

8.3 Conclusions

The objective of this thesis was to assess the impacts of group B herbicides on the nitrogen fixation of grain and pasture legumes grown in southern Australia, and determine the mechanisms by which such effects were mediated. It was found that recommended group B herbicides do have the potential to reduce the amount of nitrogen fixed by plants. This was principally related to reductions in plant growth, although more specific mechanisms involving the inhibition of nodulation and effects on the rhizobia remain a possibility.

Since nitrogen fixation is regulated in a number of ways and group B herbicides have multiple biochemical and morphological effects on legumes, it is likely that there is more than one mechanism by which group B herbicides affect nitrogen fixation. At the most simple level, a reduction in growth together with a tight coupling of N_2 fixation will reduce the amount of nitrogen fixed. More specific morphological effects include abnormal root hairs and disruption of meristems with consequences on infection by rhizobia, nodule initiation and nodule development. Biochemical effects in the plant include changes to nitrogen metabolism and stress responses which are likely to invoke down regulation of nitrogen fixation. The perturbation to amino acid pools may have a more direct effect on nitrogen fixation through the cycling of N and other regulatory mechanisms.

The possible role of rhizobia in the susceptibility of the symbiosis to herbicide is yet to be resolved. Some reported studies using mutant rhizobia suggest that if the BCAA pathway in rhizobia is disrupted, the ability of rhizobia to infect and nodulate will be compromised. There is some evidence that even if a symbiosis is successfully established, bacteroids become dependent on the plant for branched chain amino acids. Thus if BCAA production in the plant is inhibited by herbicide, bacteroids are unlikely to function although other evidence suggests that high specific activities of bacteroid AHAS may actually assist symbiotic plants.

Numerous effects of AHAS inhibiting herbicides on the plant, the rhizobia and the interactions between them can therefore be anticipated. This is consistent with the variability in the response of growth, nodulation and nitrogen fixation of grain and pasture legumes to the various group B herbicides. Climatic and edaphic factors affect

the availability and persistence of herbicide and impose additional stresses on the plant. Thus the impact of group B herbicides on nitrogen fixation in the field is subject to a number of complex factors that are yet to be satisfactorily elucidated.

8.4 Implications

Along with the uncertainties related to the regulation of nitrogen fixation and the chronic effects of group B herbicides on plants, there is much variability in the tolerance of legumes to the different herbicides, and also the climatic and edaphic factors that influence the severity of the effects. It is therefore not possible to make new recommendations regarding the application of group B herbicides to legume crops and pastures. The comparison of Herald and FEH-1 cultivars of *Medicago littoralis* showed that the severity of the reduction in nitrogen fixation is at least partially related to the susceptibility of the plant to the herbicide. Thus if symptoms such as leaf yellowing and growth inhibition are observed it is likely that nitrogen fixation has been adversely affected. This work shows that group B herbicides can inhibit nitrogen fixation. In future herbicide assessment trials, more attention should be paid to nodulation and nitrogen fixation and the results appropriately reflected in herbicide label recommendations. Herbicide labels should be strictly adhered to, and any symptoms should be treated as indicating reduced N_2 fixation. One practical recommendation to farmers would be to leave an unsprayed strip to allow simple comparisons of nodulation and leaf yellowing in the absence and presence of herbicides to investigate whether nitrogen fixation has been affected in a particular legume crop or pasture. Herbicide application (which herbicide, when applied, how much) or cropping regime (variety, fertilisation) could be altered by the farmer, taking into account the site specific climatic and edaphic influences to minimise or compensate for the potential influence of herbicides on N_2 fixation. Adoption of herbicide tolerant cultivars such as 'Angel (FEH-1) will also alleviate the problem.

8.5 Future work

The work described in this thesis has identified numerous avenues for further exploration to improve the understanding of the mechanisms by which group B herbicides affect N_2 fixation and the implications for farm practice and soil N fertility.

The biochemical pathways identified as being affected by herbicide application in the proteomics work present some specific areas for further investigation. These relate to the determination of the mechanisms of group B herbicide action, but also have application to other areas of herbicide science. Nitrogen assimilation and primary metabolic pathways (glycolysis) have been investigated by other researchers. However, further understanding of the role of specific glutathione-S-transferases (GSTs) in determining the tolerance of particular legumes to particular herbicides will have a number of applications: GSTs can be explored as a mode of tolerance of legumes to group B herbicides; if a relationship between GST expression profiles and the susceptibility of legume varieties or the potency of herbicides could be established, GSTs could be used as markers for selection of herbicide tolerant varieties with the potential for genetic modification and provide additional herbicide tolerance information in variety trials; if herbicide specific GSTs are discovered, they may have potential use in bioassays for the presence and amount of particular herbicides in soil.

In Chapter 7, some observations of root growth and morphology were made using medic plants grown on agar plates. A more detailed experiment to quantify the effects of herbicides at a range of concentrations (including concentrations relevant to the field) on root hairs and meristems would be worthwhile. To explore the importance of the impact of group B herbicides on roots hairs and subsequent infection by rhizobia, the effects of metosulam on crack entry in lupins could be used (J. Howieson, pers. comm.).

The screening experiments used group B herbicides recommended for application to legume crops and pastures. The timing (growth stage) and method of application (post sowing pre-emergence to the soil, or with agricultural nozzles in a spray cabinet) was also as recommended. For many experiments, herbicide application occurred well after root hair development, infection and nodule development had commenced and thus the potential effects on nitrogen fixation may have been missed. In addition, sulfonylurea herbicides are known to persist in the soil and as such can interfere with all stages of the development of symbioses. Thus the timing of herbicide application and the implications for the various interactions that may occur with saprophytic rhizobia, root hair infection, plant growth, nodulation and nitrogen fixation itself deserves further attention. To address these concerns, a longitudinal time course study including

acetylene reduction assays and measurement of nodulation over a range of time points would be a very useful experiment.

Further work is required to determine whether sub lethal herbicide effects on rhizobial function occur. There are a number of ways to do this:

Firstly an assay of AHAS enzyme activity in inoculant strains will identify whether the herbicides have the potential to directly affect rhizobia. Most studies are limited to assessing the growth of rhizobia, however it is possible to have physiological effects that do not manifest in altered growth rates. Assays of extracted AHAS enzymes from rhizobia have shown that similar inhibition occurs as observed for plant enzymes (Royuela *et al*., 1998).

As well as assays of rhizobial AHAS, the most probable number (MPN) technique could clarify impacts of the group B herbicides on the nodulation capacity of rhizobia. A range of herbicide concentrations up to saturation should be used since herbicide in soil water can be more concentrated than that calculated on a soil volume basis. By using herbicide tolerant legumes such as FEH-1 it will be possible to address any issues of herbicide carry over affecting the plant.

The proteomics work on *Medicago* truncatula identified a number of root proteins whose relative abundance was changed by exposure to herbicide. A proteomic study could be conducted on rhizobia (Djordevic *et al*., 2003) to determine what effects, if any, the herbicides have on the microsymbiont. This would be one way to determine whether physiological effects occur even though the growth of rhizobia appears to be unaffected. It is also possible to perform proteomic analysis on bacteroids (Djordevic, 2004).

It has been proposed that legumes in symbiosis are more tolerant to group B herbicides because of the high specific activity of AHAS in bacteroids (Royuela *et al*., 1998; Zawoznik and Tomaro, 2005). Presumably this involves the transport of branched chain amino acids or intermediates from the bacteroids to the plant. Amino acid cycling is emerging as a way in which fixed nitrogen is transported from bacteroids to the

legume. Further investigation into the enhanced tolerance of symbiotic legumes to herbicide and the transfer of fixed N between symbionts via amino acid cycling is required. This will complement research into the importance of branched chain amino acids in particular for symbiosis. Functioning BCAA synthesis in *Sinorhizobium meliloti* is required for nodulation of *Medicago sativa*, but other symbioses may have different requirements.

As well as providing nitrogen through symbiotic nitrogen fixation, soil associated microorganisms can have other plant growth promoting (PGP) activity. Ryu *et al*. (2003) assessed plant growth promotion of *Arabidopsis* by volatile compounds produced by various PGP rhizobacteria. Acetoin and 2-3-butanediol were produced exclusively by the two strains of *Bacillus* that elicited the greatest growth promotion in Arabidopsis in that study. *Bacillus* with insertional knockouts of ALS and ALD operons did not promote plant growth. Acetoin is produced from acetolactate. It is expected that this acetolactate is produced by catabolic acetolactate synthase and is therefore not susceptible to herbicide. To test this hypothesis a PGP assay could be used to determine whether group B herbicides affect plant growth promotion by PGP bacteria.

In contrast to the in-crop group B herbicides, the sulfonylureas (SUs) are not recommended for direct application onto legume crops and pastures. The SUs are highly potent and persist in the soil, particularly in dry conditions and high pH. Persistent SUs are likely to have different effects on the symbiosis compared to the recommended in-crop group B herbicides since saprophytic bacteria are more likely to be exposed to SUs, SUs are more likely to come into direct contact with roots, and the herbicide will be present throughout the nodulation process. The distribution of SUs in the soil profile and the concentrations available will be highly variable due to conditions required to break them down. A screening experiment using residual concentrations of SUs will indicate the susceptibility of different legumes and complement the screening experiments conducted on recommended in-crop group B herbicides in this thesis. MPNs on rhizobia in soils with residual SUs will indicate whether the ability of rhizobia to form symbioses has been compromised.

The comparison of FEH-1 *Medicago littoralis* with Herald showed the potential for herbicide tolerant varieties to address reduced levels of N_2 fixation caused by group B herbicides. This herbicide tolerant variety was produced by chemical mutagenesis, thus multiple mutations may have occurred. Through a targeted approach a single mutation in the *ALS* gene was found (Oldach *et al*., 2008) and implicated as the mechanism of herbicide tolerance. In Chapter 6, where no herbicide was applied in the glasshouse experiment, Herald had higher levels of nodulation and nitrogen fixation than FEH-1. This would indicate that in the absence of any history of group B herbicide application, Herald may remain the better choice of cultivar. A quantitative assessment of nitrogen fixation by the two cultivars in the field would confirm whether farmers can expect similar or improved levels of nitrogen fixation. A proteomic comparison of FEH-1 and Herald may also identify important pathways of herbicide tolerance and implications for nitrogen fixation.

Reply to examiners reports

I am very grateful for the thorough and constructive examination of my thesis.

6 issues, summarised below, were identified as requiring further attention:

1) No treatment of proteomics in the literature review.
2) Consider splitting proteomics chapter so that the root and root hair results are not buried.
3) Why was *Medicago truncatula* used in the proteomics work instead of Herald and FEH-1, for example?
4) In chapter 6, why was the effects on optical density presented as an average over 30 and 42 hours?
5) Crack entry with lupins. Coverage in the literature review on how infection by crack and root-hair entry differ.
6) Future work – timing of herbicide application. The need for a longitudinal time-course study including acetylene reduction assays over a number of time points.

Each of these points are now addressed below:

1) No treatment of proteomics was given in the literature review because at the commencement of candidature, when the literature review was undertaken, there was no expectation that a proteomics study would be conducted. I did search the literature at the commencement of the proteomics work, prior to submitting my thesis and again on receiving the examiners reports. In the introduction of Chapter 7, my attempts to review the relevant literature are summarised in the penultimate paragraph. Proteomics was still in its infancy and there were no examples in the literature with direct relevance to the application of proteomics to study the effects of herbicides on legumes in particular. This is still the case today and I therefore there is no additional information to be included.

2) The root and root hair results came from observations that were made whilst conducting the proteomics experiments. They were initially not intended to have been included in the thesis, but as one examiner pointed out the

observations turned out to be quite important. I agree that it would have been preferable to include the root work as a separate chapter from the proteomics work, and had a more rigorous study been conducted with a range of herbicide concentrations that would be inclusive of conditions expected to occur in the field, this certainly would have been a separate chapter. However I feel that standing alone, the root observations are somewhat weakened in that a systematic quantification of root hair development was not made and the herbicide concentrations used are probably too high to be relevant to field grown legumes. Based on my observations, a more detailed study would be recommended, and has been included in the suggestions for future work. However I think the observations are better used as supporting information as is currently the case in Chapter 7. In addition, if a stand alone chapter based on the root and root hair observations was created, the criticism of why *Medicago truncatula* was chosen would be more difficult to defend. As it currently sits there is justification because it was linked to the proteomics work. Another general comment made was about the distinction between results and discussion. This comment was especially poignant for chapter 7, where I put the photographs of root hairs and tips in the discussion rather than the results. This was done because no quantitative assessments were made and thus the observations provide supporting information to inform the discussion, rather than a solid set of stand-alone results. I acknowledge the examiner's comments on how 'stark' the results were and how they seem to be 'key experiments.' It was not without deliberation that I presented it as submitted for examination. However, I have rearranged the results and discussion on the strong advice of the examiner.

3) As acknowledged by the examiner, *Medicago truncatula* was used for the proteomics study because its proteome had already been quite well characterised by the group at RSBS and the existence of extensive databases enabled identification of differentially expressed proteins. Thus there was a strong methodological preference to use *M. truncatula*. In addition, this collaborative work was partially funded by RSBS and ARC Centre of Excellence for Integrative Legume Research. There was a strong preference to use *M. truncatula* as it fitted in with other work they were doing. One other thing to

consider is that the proteomics work was actually conducted prior to the FEH-1 work, but is presented in the other order in the thesis because I felt the flow of ideas and information was better. At the time of the proteomics work, FEH-1 was still commercially sensitive and I had received seed in good faith that it not be distributed elsewhere. I agree that it would have been interesting and probably more relevant to conduct a proteomics study on FEH-1 and Herald. This in fact was considered prior to and after going to RSBS but was decided against for the above mentioned reasons. I believe it would still be an interesting study to conduct, particularly because I am unconvinced that the single mutation identified by Oldach et al (2008) does not satisfactorily explain some of the other differences I have observed between FEH-1 and Herald. A statement has been included in the future work section of the final discussion.

4) In the ANOVA including time and herbicide as factors, there was only a main effect of herbicide on A500 and hence the average of 30 and 42 hours was presented. I had considered doing a more detailed time course experiment to address growth effects on rhizobia in cultures but went down the path of trying to develop an *in* vivo assay for AHAS activity in rhizobia. Essentially I ran out of time and to my thinking, the rhizobia question is still in an unsatisfactory state.

5) I must admit that crack entry was not given much consideration during my candidature. It was included more explicitly in the literature review after one of my reviewers requested it. The examiner is correct in that crack entry could be another way to tease out potential mechanisms by which herbicides effect nodulation and hence nitrogen fixation. This has been noted in the future work section but am uncomfortable giving crack entry more attention in the literature review because it was not part of my thinking during my candidature.

6) I had given a lot of thought to timing of herbicide application and the implications for the various interactions that may occur with saprophytic rhizobia, root hair infection, plant growth, nodulation and nitrogen fixation itself. I think my experiments addressed this to some extent, particularly with inclusion of post-sowing pre-emergence herbicide applications in the screening

> experiments. However I agree that in future this can be further addressed with a more detailed time course study and had included this as a suggestion for future work in earlier drafts of my thesis, particularly with regards to sulfonylurea herbicides. It must have been dropped during restructuring chapters and I have now reinstated it in the future work section.

Again, I thank the examiner for the thorough assessment of my thesis and constructive comments.

I also acknowledge and thank an anonymous examiner for their efforts in ensuring my thesis is of international academic standard.

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