THE EFFECT OF HERBICIDES ON N_2 FIXATION IN FIELD PEA (*PISUM SATIVUM* L.) AND CHICKPEA (*CICER ARIETINUM* L.)

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ABSTRACT

The use of herbicides in cropping systems is routine in western Canada as is the practice of rotating crops between cereals, oilseeds and pulse crops. Often, herbicides that are appropriate one year in the crop rotation are not compatible with the following crop. Additionally, certain herbicides are designed to target certain enzyme pathways that can interfere with amino acid synthesis. These pathways also exist in the microbial community, including *Rhizobium* species. Rhizobia have a unique symbiotic relationship with legumes. In return for a carbon source, rhizobia not only fix atmospheric dinitrogen (N_2) for the plant, but also can increase soil N reserves for the following year. With herbicides targeting amino acid synthesis in both plants and microbes, there is a possibility that N_2 fixation may be inhibited by the application of certain herbicides.

This project was designed to examine possible negative effects of herbicide application on N_2 fixation in field pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.). The study included field, growth chamber and laboratory experiments in which the effects of pre- and post-emergent herbicides, as well as herbicide residues in soil were examined.

In the field experiments, some early season measurements suggested that herbicide application had a negative impact on various growth and N_2 fixation parameters. However, as the season progressed, plants recovered from early herbicide damage and N_2 fixation ultimately was relatively unaffected. Growth chamber experiments similarly revealed that N_2 fixation was largely unaffected by herbicide application when the application rates were relatively low (i.e., at rates intended to simulate partial herbicide breakdown, and thus lower than the recommended field rate). Although, N_2 fixation was suppressed where high rates of herbicide (i.e., greater than recommended field rate) were applied, the efficiency of the rhizobia to fix N_2, (i.e., the amount of N_2 fixed per unit nodule mass), was unaffected. This along with a laboratory experiment which monitored growth of rhizobia *in vitro*, confirmed that rhizobia were not directly affected by the herbicides used in this study and that overall N_2 fixation was not inhibited directly by the application of these herbicides. It was concluded that any negative

impact on N_2 fixation caused by herbicides used in this study, was related to the impact of the herbicide on crop growth, and was not due to any direct effects of the herbicide on the rhizobia.

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ACCase	Acetyl-CoA-carboxylase
a.i.	Active ingredient
AHAS	Acetohydroxy acid synthase
ALS	Acetolactate synthase
ANOVA	Analysis of variance
ARA	Acetylene reduction assay
BNF	Biological N_2 fixation
DAA	Days after application
DAE	Days after emergence
DAP	Days after planting
EC	Electrical conductivity
ECW	Expert Committee on Weeds
EDC	Endocrine disrupting chemicals
EPSP	5-enolpyruvaylshikimic acid 3-phosphate synthase
GC	Gas chromotography
IAA	Indole-acetic acid
MCPA	2-methyl-4-chlorophenoxy acetic acid
MCPB	4-(4-chloro-o-tolyloxy) butric acid
N_2	Di-nitrogen
NA	Nitrogenase activity
Ndfa	Nitrogen derived from the atmosphere
NS	Not signficant
PPT	Phosphinothricintripeptide
PT	Phosphinothricin
RCBD	Randomized complete block design
YEM	Yeast extract mannitol
YMB	Yeast mannitol broth
2,4-D	Dichlorophenoxyacetic acid

1 INTRODUCTION

In 2006, Saskatchewan growers planted over 1.8 million hectares of pulse crops (Saskatchewan Pulse Growers, 2007) which accounted for 78% of Canadian field pea production, 99% of lentil production and greater than 90% of chickpea production (Pulse Canada, 2008a). Additionally, Canadian exports of pulses exceeded $1 billion in 2006 (Pulse Canada, 2008a). Canada is now the world's largest exporter of lentil and pea, the third largest exporter of chickpea and the fifth largest dry bean exporter (Agriculture and Agri-Food Canada, 2005).

Legumes such as field pea and chickpea are high in protein and have high N requirements that generally are met through a symbiotic relationship with N_2-fixing *Rhizobium* species. However, the amount of N_2 fixed is influenced by several factors including soil type, nutritional status of the soil, crop species and variety, water availability and temperature as well as soil and crop management (Ledgard and Steele, 1992). In addition, N_2 fixation depends on the ability of the plant to provide photosynthetically-fixed carbohydrates to the rhizobial partner (Bergersen, 1982; Mårtensson, 1992; Yoneyama, 2005). Thus, any factor or factors that influence this relationship may have a negative impact on the N_2-fixing association and consequently the N supply to the plant. For example, the chemicals presently used in agriculture may shift and disrupt microbial communities in the soil and therefore affect the symbiotic relationship between N_2-fixers and legumes (Flores and Barbachano, 1992).

The common use of herbicides in agriculture may negatively affect N_2 fixation, either directly by affecting the rhizobia (Mallik and Tesfai, 1985; Anderson et al., 2004), or indirectly by reducing photosynthate allocation to the nodules for N_2 fixation (Sprout et al., 1992; Koopman et al., 1995) or by restricting root growth and hence the number of root sites available for infection (Eberbach and Douglas, 1991). Additionally, herbicides that are persistent in the soil may have a long-lasting impact on rhizobial survival and function (Eberbach and Douglas, 1989; Mårtensson and Nilsson, 1989; Koopman et al., 1995; Eliason et al., 2004).

While there has been some research on the effect of herbicides on N_2 fixation in pulse crops, few studies have been conducted in western Canada and most of these

studies have examined a limited range of herbicides (e.g., chloramben, linuron, trifluralin, diclofop and metribuzin) (Rennie and Dubetz, 1984; Sprout et al., 1992). By scrutinizing the impact of herbicides on N_2 fixation and the consequent crop yield, as well as the mechanisms by which herbicides may affect nodulation and subsequent nodule occupancy, we can begin to develop effective strategies to minimize the impact of herbicides on the N_2-fixing association.

The objectives of the research described in this thesis were to:

1. Assess the impact of pre- and post-emergent herbicides used for weed control in field pea and chickpea on nodulation, N_2 fixation and consequent yield.

2. Assess the impact of herbicides, known to have soil residual properties and used in crops preceding field pea, on nodulation, N_2 fixation and consequent yield.

3. To compare the use of inoculation types (i.e., granular soil-applied inoculant versus peat-powder seed-applied inoculant) on N_2 fixation by pea subject to herbicide stress and to determine if inoculation or inoculant formulation influences the impact of herbicides on N_2 fixation.

2 LITERATURE REVIEW

Field pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) are grown in crop rotations in Saskatchewan. Including pulses in rotations that consist of cereals and oilseeds, may be beneficial for several reasons including breaking the disease cycle and increasing available N in the soil for succeeding crops. Field pea and chickpea are both ground for flour (Oplinger et al., 1990; Oelke et al., 1991), but most pea is exported for animal feed (Pulse Canada, 2008b). Field pea has high levels of essential amino acids including lysine and tryptophan which are usually low in cereals; therefore, field pea is included as a protein supplement in livestock feed (Oelke et al., 1991). Both crops are adapted as cool season crops and grow on soils that are well drained – an excellent combination for cropping in Saskatchewan.

In most cropping systems, N is a major nutrient requirement and is needed for amino acid production which is used to assemble proteins and nucleic acids (Tisdale et al., 1993). Nitrogen is also required in carbohydrate utilization and is contained in enzymes known to stimulate root development and activity (Olson and Kurtz, 1982). Although chickpea and field pea are known to be high protein crops, most of the N requirement is met through symbiotic N_2 fixation. Moreover, when grown in association with an appropriate *Rhizobium* partner, these crops have the potential to increase the amount of soil N in the subsequent year and thus can reduce fertilizer requirements (Walley et al., 2007). It is essential that the legume-*Rhizobium* association be enhanced in order to maximize N_2 fixation and its potential benefits in crop rotations.

Field pea and chickpea have a specific association with bacteria capable of fixing N_2, namely *Rhizobium leguminosarum* and *Mesorhizobium ciceri*, respectively. Under ideal conditions, rhizobia can provide between 60 to 80% of the required N from the atmosphere (Saskatchewan Agriculture and Food, 2001, 2002). Field pea typically supports effective N_2-fixing associations whereas chickpea are not considered strong N_2 fixers even when an adequate number of nodules is present (Beck et al., 1991).

Several conditions are required to promote the stepwise development of an effective

N_2-fixing association (Anderson et al., 2004). First, there must be the appropriate rhizobia species in sufficient numbers either present in the soil or inoculated with the seed. If sufficient numbers of viable rhizobia are present combined with a favourable environment, complex signaling between the plant and the rhizobia occur to initiate nodulation. The rhizobia first attaches to a root hair, then the bacteria alters the epidermal hairs on the root such that the root hairs curl back on themselves and trap the bacteria within the fold (Yao and Vincent, 1969). An infection thread then begins to develop. Meristematic growth is induced by the rhizobia and the infection thread carries the bacteria into the meristem from which a nodule develops (Rossen et al., 1985). Within this nodule, rhizobia convert N_2 to NH_3 – a plant available form of N. Each of these steps can be influenced directly or indirectly by external environmental factors such as the application of agrichemicals.

2.1 Biological N_2 Fixation

Because N is the most frequently deficient nutrient in crop production and as the cost for N fertilizer increases, biological N_2 fixation (BNF) is increasingly important as an environmentally sustainable way of enhancing soil N reserves and providing legumes and following crops with N from the atmosphere instead of the soil (Peoples et al., 1995). It is estimated that on a global scale, BNF may provide as much as 175 million metric tons of N per year (Hubbell and Kidder, 2003). Preserving the relationship between rhizobia and plants will continue to be important as world population and requirements for food production increase.

2.2 Inoculation and Inoculant Formulations

Given the importance of the legume-*Rhizobium* association, inoculants containing *Rhizobium* bacteria are commonly used in pulse crop production. Inoculant formulations include seed applied products that use agar, broth (concentrated and frozen), oil, and peat as a carrier (Date, 2001). In Saskatchewan, seed inoculants are normally applied in a liquid formulation or as a peat-based powder (Saskatchewan Agriculture and Food, 2007). However, not all inoculants are applied to the seed directly. In some situations seed application of rhizobia may be unproductive if the seed is pretreated with a pesticide that is incompatible with rhizobia (Brockwell and

Bottomley, 1995). Fortunately, there are other application and placement options. Rhizobial inoculants may also be purchased as a "soil implant" intended to be placed in the furrow at the same time as the legume seed. Another method of inoculant application includes a post-emergent treatment or as an inclusion in furrow irrigation (Brockwell and Bottomley, 1995).

Several studies have compared inoculant formulations to determine differences in efficacy of the products (Sprout et al., 1992; Kyei-Boahen, 2000; Kyei-Boahen et al., 2002). Many of these studies have attributed differences in efficacy to nodule placement. Specifically, mobility of rhizobia in the rhizosphere and soil is limited, especially in drier soils found in Saskatchewan, and nodule formation may be restricted to the point of inoculation (Wadisirisuk et al., 1989). Therefore seed inoculation using either a liquid formulation or a peat-based powder as a carrier for the rhizobia results largely in crown nodulation. Soil implant inoculation (also known as soil inoculation) results in nodules being distributed over a greater portion of the root system (Ciafardini and Barbieri, 1987; Kyei-Boahen et al., 2002).

Kyei-Boahen et al. (2002) and Clayton et al. (2004) compared various inoculant formulations and reported that granular inoculation both enhanced nodule frequency and consequent yields. Hardarson et al. (1989) compared seed and soil inoculants in a growth chamber experiment. Their results agree with Kyei-Boahen et al. (2002) and Clayton et al. (2004). With seed inoculation, most of the nodules occurred on the crown region of the roots, whereas granular inoculants typically resulted in nodules which were more numerous and well distributed throughout the root. These results suggest that granular inoculants are particularly well suited for Saskatchewan. Moreover, it is possible that different inoculant formulations may promote higher N_2 fixation in combination with the use of herbicides by increasing nodule number and distribution.

2.3 Herbicides

In addition to inoculants, herbicides are also commonly used in pulse crop production. The role of herbicides is to reduce competition with the crop by undesirable weed species. Herbicides attack certain target sites and can alter cell metabolism within the unwanted plant (Vidal et al., 1992). Target sites include enzymes, proteins, or other pathways in the plant where the herbicide may bind and disrupt normal plant functions.

Importantly, some microbes also share similar potential target sites (Burnet and Hodgson, 1991; Royuela et al., 1998; Zablotowicz and Reddy, 2004). For example, the enzyme acetolactate synthase (ALS, also called acetohydroxy acid synthase [AHAS]) is involved in synthesizing branched-chain amino acids in both plants and rhizobia (Royuela et al., 1998). Some classes of herbicide (e.g., sulfonylureas, imidazolinones and pyrimidinyloxybenzoates) bind to this enzyme and cause a malfunction of the enzyme that in turn reduces the synthesis of amino acids necessary for protein synthesis (Prather et al., 2000), effectively causing the plant to starve. Similarly, the herbicide glyphosate inhibits the enzyme 5-enolpyruvaylshikimic acid 3-phosphate synthase (EPSP), in the shikimic acid pathway of target weeds, which inhibits the synthesis of amino acids. Rhizobia share a glyphosate-sensitive enzyme and in greenhouse and field experiments, Zablotowicz and Reddy (2004) found that nodulation and nodule leghemoglobin content was inhibited by glyphosate applications.

The possibility that herbicides may affect microbes raises concerns when applying herbicides to legume crops. Although herbicide application may be necessary because weeds negatively affect crop production, application may limit the efficiency of N_2 fixation (De Felipe et al., 1987). Therefore the use of herbicides is appropriate only if they do not interfere with N_2 fixation. For example, Atlas et al. (1978) conducted a growth chamber experiment using soybean (*Glycine max* L.) and found that when herbicides were applied at their field rate, there were no detectable adverse effects. In other cases, it has been reported that triazines may enhance N_2 fixation in the legume-*Rhizobium* symbiosis. Simazine has stimulative properties that are attributed to its action on nitrate reductase activity (Tweedy and Ries, 1967; Wu et al., 1971, 1972).

Anderson et al. (2004) claim that herbicides may affect the legume-*Rhizobium* relationship by: (1) affecting the host plant (i.e., there may be a reduction in root biomass that leads to fewer infection sites or by affecting the carbohydrate supply to existing nodules); (2) affecting rhizobial survival or growth that leads to a decreased potential for rhizobial infection on root hairs; (3) inhibiting or inactivating the biochemical signaling that plants require to initiate nodule development – this inhibition could affect rhizobia or plants; and (4) inhibiting nodule development by reducing the capacity for cell division.

There are three possible herbicide application methods: pre-plant incorporated, pre-emergent and post-emergent. Pre-plant incorporated herbicides include seed-applied pesticides that in some cases can retard nodulation, particularly on the primary roots (Dunigan et al., 1972; Goring and Laskowski, 1982). However, Goring and Laskowski (1982) reported that there was little permanent effect on nodulation of lateral roots. Similarly, pre-emergent herbicides are more likely to have direct contact with seed-applied or soil rhizobia which could lead to deleterious effects, whereas post-emergent herbicides are less likely to affect nodulation directly (Rennie and Dubetz, 1984). For example, according to Sprout et al. (1992), there were detrimental affects on *R. leguminosarum* by the post-emergent herbicide metribuzin. They concluded that this was due to direct negative effects on the plant, which resulted in indirect effects on nodulation and N_2 fixation.

2.3.1 Direct effects of herbicides on host plants

Herbicides often reduce not only protein and amino acid production, but also carbon (C) fixation by plants and thus decrease the proportion of C allocated for the synthesis of secondary compounds (Daniel et al., 1999). Because of this, shoot and root growth may be affected. It is possible that a plant with restricted root growth may have a reduced number of root sites available for rhizobial infection (Eberbach and Douglas, 1991). Symbiotic N_2 fixation depends on the attachment of rhizobia to the root in order to receive C energy derived from photosynthesis (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977). If the plant cannot provide a photosynthate supply to rhizobia, N_2 fixation is likely to be negatively affected.

As an example, Gupta (2005) found that the herbicide flumetsulam, applied to 6 wk old pea caused some yellowing of shoots and a significant reduction in plant growth and the number of effective nodules. There was a partial recovery in the nodules 4 to 5 wk after the herbicide application, but the contribution of these nodules to overall N_2 fixation may have been limited because of the earlier stress on the plants.

Sprout et al. (1992) found that metribuzin applied 8 d after planting (DAP) to pea plants grown in Leonard jars decreased root biomass, plant weight, number of nodules and C_2H_2 reduction (a measure of N_2-fixing activity). They observed, that at 8 DAP, reduced plant biomass decreased available photosynthate which coincided with an

inhibition of initial nodule development. They concluded that metribuzin treatments primarily affected plant growth rather than *R. leguminosarum* activity. These detrimental effects of photosynthesis-inhibiting herbicides are supported by other research on inoculated and non-inoculated legumes (e.g., Bertholet and Clark, 1985; Knott, 1987).

Sprout et al. (1992) observed that the degree of inhibition of plant growth observed with metribuzin was dependent on several factors. These included the time of herbicide application, time of sampling after herbicide application and the *R. leguminosarum* strain used for inoculation. In their experiment, metribuzin, applied 13 DAP, had little effect on plant growth and development of the legume-*Rhizobium* symbiosis. They suggested because older plants have a higher rate of photosynthesis, the metribuzin application did not interrupt advanced photosynthesis. An earlier herbicide application, occurring when nodulation was initiated, may cause a decrease in the available photosynthate that would hinder plant growth and subsequent nodule development.

In contrast, Jensen (1993) found that when tank mixes of cyanazine with either bentazon, bentazon/MCPA or MCPB were applied at the first node stage in field pea before the first leaf unfolded, there was little damage. They concluded that later treatments interfere with reproductive development in the plant and were therefore more detrimental to yield than early treatments that did not interfere with the reproductive development or the grain-filling period.

In a field study, Rennie and Dubetz (1984) reported that metribuzin applied as a pre-emergent treatment at 0.4 kg ha^{-1} on soybean decreased germination and plant growth, but N_2 fixation estimated using the acetylene reduction assay (ARA) was higher in herbicide treated plants as compared to the untreated control. They determined that the effect of metribuzin was not on nodulation or nitrogenase activity (NA) but rather on plant growth. These studies collectively suggest that it is the timing of herbicide application that negatively affects plant growth thereby limiting photosynthate availability and consequent N_2 fixation.

2.3.2 Effect of herbicides on rhizobial survival and growth

While herbicides may directly affect plant growth and thus indirectly affect rhizobia and N_2 fixation, herbicides also may have an impact on rhizobial survival and growth.

The application of herbicides in agricultural systems may exert side effects on the soil microflora, including a possible shift in microorganism community structure (Burnet and Hodgson, 1991; Boldt and Jacobsen, 1998; Royuela et al., 1998). This may be particularly true in the case of compounds which interfere with amino acid biosynthesis and therefore also may affect microbial metabolism (Royuela et al., 1998; Prather et al., 2000; Zablotowicz and Reddy, 2004).

Anderson et al. (2004) pre-exposed rhizobia to chlorsulfuron before inoculating them into pots with germinating chickpea seeds. Pre-exposure reduced the number of nodules formed by 51%. In the lab under sterile conditions, Kriete and Broer (1996) concluded that *R. meliloti* is sensitive to low concentrations of the herbicide phosphinothricintripeptide (PPT) and its active ingredient phosphinothricin (PT). However, they also noted that in non-sterile soil, nodulation and N_2 fixation rates were not changed by the herbicide – perhaps because of the rapid degradation and or absorption of PTT and PT in the soil.

Under sterile lab conditions, Moorman et al. (1992) grew *Bradyrhizobium japonicum* culture and mixed it directly with glyphosate at 0, 0.5, 1.0 and 5.0 mM. They plated the solution on yeast extract mannitol (YEM) agar (1, 2 and 3 d after inoculation). The 0.5 mM glyphosate level led to a gradual decline in cell numbers, whereas the lower concentrations allowed some growth. All levels of glyphosate had a lethal effect on *B. japonicum* in a free-living state, i.e., in broth culture. Santos et al. (2005) similarly examined the tolerance of various *Bradyrhizobium* strains to glyphosate formulations. They inoculated YEM broth containing 43.2 µg L^{-1}a.i. of glyphosate and found that growth of 21 out of 24 strains was reduced compared to the control. King et al. (2001) had a controlled greenhouse study examining growth and NA of glyphosate tolerant soybean. Although early applications of glyphosate generally delayed N_2 fixation and decreased root and shoot biomass and N accumulation in soybean harvested at 19 d after emergence (DAE), plants recovered by 40 DAE (King et al., 2001).

In New Zealand, Clark and Mahanty (1991) examined the effect of herbicides on *R. trifolii* growth around filter paper dipped in herbicides and placed on agar plates, as well as in broth culture and in growth chamber experiments. On agar plates, rhizobial growth was inhibited by all herbicides (paraquat, MCPB, bentazon, propyzamide and

fluazifop-p) at the 1× rate and the inhibition zone increased as the rate increased to 10× the recommended rate. However, in broth culture, rhizobial growth was unaffected, leading them to suspect that on the agar plates there was a localized concentration on the agar plates, whereas in the broth culture the herbicide was more homogeneous. In their growth chamber experiment, only paraquat decreased biomass, but all herbicides resulted in a decrease in nodulation. Overall, white clover (*Trifolium repens* L.) biomass was unaffected although nodulation was decreased.

Singh and Wright (2002) examined the impact of herbicides on rhizobial growth by monitoring optical densities of broth cultures. Specifically they inoculated rhizobia into terbutryn/terbuthylazine, trietazine/simazine, prometryn and bentazon solutions. Terbutryn/terbuthylazine (>4.0 mg L^{-1}), trietazine/simazine (>4.3 mg L^{-1}), and prometryn all caused decreased rhizobial growth; but bentazon had no effect.

In studies that included sterile lab experiments in conjunction with growth chamber or field experiments (Eberbach and Douglas, 1991; Singh and Wright, 1999; Singh and Wright, 2002), most studies revealed that without soil as a buffer, growth and survival of rhizobia were inhibited. However, when extended into the field, the results were variable. In some experiments, herbicide application initially reduced N_2 fixation, but later in the season, plants recovered and no differences were detected. It should be noted that although rhizobia are affected in sterile and free-living states, these results ought not to be extrapolated to the field.

2.3.3 Effect of herbicides on biochemical signaling in rhizobia and nodule initiation and development

Some studies indicate that there are direct herbicidal effects on rhizobia (Clark and Mahanty, 1991; Moorman et al., 1992; Royuela et al., 1998; Prather et al., 2000; Zablotowicz and Reddy, 2004). Assuming no direct effects on growth and survival, there may be an opportunity for a negative effect on chemical signaling between plants and rhizobia. The establishment of an effective N_2-fixing association between an appropriate *Rhizobium* bacteria and a host plant is regulated by a series of biochemical signals from both partners. In rhizobia, nodulation genes are switched on in the presence of an acceptable host – roots that release exudates that include lectins, flavonoids and/or isoflavonoids (transmembrane receptors) and nutrients such as organic

acids (Garg and Geetanjali, 2007). These ultimately control the initiation of nodule formation (Long, 1996; Garg and Geetanjali, 2007).

There is an order of events that regulates the signaling between rhizobial nodulation genes and the host plant. The first occurs between the root-hair and bacterium. Four *R. leguminosarum* genes are involved in the induction of normal root-hair curling which initiates nodulation (Rossen et al., 1984). This involves precise regulation of bacterial and plant genes (Rossen et al., 1985). Herbicides may affect these events by interrupting molecular interaction processes which lead to the recognition, binding and penetration of rhizobia in leguminous roots (Flores and Barbachano, 1992; Musarrat and Haseeb, 2000).

Rhizobia are usually found within the top 25 cm of the soil profile and it is within this soil environment that endocrine-disrupting chemicals (EDC) may affect phytoestrogen signaling and thus N_2-fixing symbiosis (Fox et al., 2001; 2004). Plants produce phytoestrogens to deter herbivores, as attractant cues for insects and as recruitment signals for symbiotic soil bacteria (Fox et al., 2004). Because phytoestrogens are integral for recruiting *Sinorhizobium meliloti*, Fox et al. (2004) tested whether EDCs block the critical phytoestrogen signaling system that regulates symbiosis between plants and bacteria. In their study, they found that 45 of the 62 EDCs and organochlorine pesticides significantly inhibited luteolin-NodD receptor signaling and symbiotic nodule gene activations.

Daniel et al. (1999) agreed that herbicide application reduces the total amount, and alters the production levels, of multiple phytochemicals in treated plants. This is important because the amount and exact profile of phytochemicals produced by a plant directly correlates with its ability to signal and recruit symbiotic soil bacteria (Long, 1989; Daniel et al., 1999). Any alteration of the ability of the plant to signal and recruit rhizobia may inhibit signaling necessary for N_2-fixing symbiosis and reduce plant yields (Musarrat and Haseeb, 2000; Fox et al., 2004).

2.3.4 Effect of herbicides on nodule initiation and development

Many studies have shown nodulation inhibition in conjunction with herbicide application (e.g., Mallik and Tesfai, 1985; Gonzalez et al., 1996; Singh and Wright, 1999; Abd-Alla et al., 2000; Anderson et al., 2004). The decrease in nodulation may be

due to the process of nodule initiation (alteration in the quality and quantity of root exudation) or an alteration of root hair morphology (De Rosa et al., 1978; Ratnayake et al., 1978; Mårtensson, 1992). This also includes production of flavonoid compounds and lectins that play an important role in the attraction and attachment of rhizobia to root hairs (Hansen, 1994). For example, Eberbach and Douglas (1991) reported that residues of dichlorophenoxyacetic acid (2,4-D) interfered with the process of nodule initiation directly. They concluded that the observed reduction in nodulation was not related directly to the reduction in the size of the root system, but was due to the process of nodule establishment. The herbicide reduced nodulation; but the nitrogenase system was unaffected. Similarly, De Rosa et al. (1978) found that trifluralin affected nodule establishment in white clover by the deformation of root hairs; however, Rennie and Dubetz (1984) in their study on soybean, found that although this herbicide may affect nodulation, there was no effect on the process of N_2 fixation in these plants.

Gonzalez et al. (1996) studied the effect of imazethapyr on *Rhizobium* growth, nodulation ability and plant growth in a growth chamber experiment. At doses of 1.73 µM imazethapyr g^{-1} soil, nodule numbers were reduced by 45% as compared to the control but nodule size was unaffected, suggesting a direct herbicidal effect on nodule initiation rather than development. They did not attribute a direct effect of imazethapyr on rhizobia because doses higher than 0.34 mM were required to affect rhizobia growth in a defined medium. While plant growth was inhibited, they determined that the effect of the herbicide on nodule initiation was greater than its influence on nodule formation.

Musarrat and Haseeb (2000), using an *in vitro* study, examined the relationship between paraquat and plant lectins (specific carbohydrate binding proteins). The model study revealed significant conformational changes in the protein structure due to extensive binding by paraquat. Thus, they concluded that herbicides such as paraquat have a higher affinity for lectins and this may interfere with the binding of rhizobia to the corresponding lectins on the root and therefore decrease N_2 fixation.

Through past research, it is clear that herbicide application can, and in some instances does, negatively affect nodulation and subsequent N_2 fixation in a variety of legumes. It is equally clear that the specific effect can vary depending on the crop variety, *Rhizobium* species and herbicide. The challenge is to identify possible

combinations that may negatively influence pulse crop productivity in Saskatchewan and implement management strategies to reduce or eliminate potential losses.

3 EFFECT OF HERBICIDE APPLICATION ON N_2 FIXATION IN FIELD PEA (*PISUM SATIVUM* L.) AND CHICKPEA (*CICER ARIETINUM* L.) UNDER FIELD CONDITIONS

3.1 Introduction

Herbicide application has become a routine practice for most crop producers in Canada. Although much research is conducted prior to the registration of a herbicide to ensure efficacy and crop safety, less research has focused on the impact of herbicides on microbial communities and specific microbial functions. For example, despite the importance of the legume-*Rhizobium* symbiotic association little is known about the impact of routine herbicide use on the success of this N_2-fixing association. Research must take into account any negative herbicide effects on overall growth, establishment and functioning of an effective symbiosis (Khan et al., 2004).

According to Anderson (2004), there are several mechanisms by which herbicides may have a negative impact on N_2 fixation. For example, herbicides can affect the host plant thereby decreasing photosynthate supply to roots. As a consequence, root growth may be limited, decreasing the number of potential sites for infection. Herbicides may also directly affect the legume-*Rhizobium* relationship by inhibiting biochemical signaling between rhizobia and plants, by inhibiting nodule development or by directly affecting rhizobial growth and survival (Anderson, 2004).

There is potential for herbicides to interfere with the legume-*Rhizobium* association when applied either as a pre- or post-emergent treatment, or if herbicide residues remain in the soil from one cropping season to the next. Timing of a herbicide application may dictate the occurrence and severity of a negative interaction in the symbiotic relationship (Rennie and Dubetz, 1984; Sprout et al., 1992; Jensen, 1993).

Pre-emergent herbicides are applied in the cropping system pre- or post- seeding to control early weed growth. These include inhibitors of carotenoid synthesis, photosynthetic inhibitors and ALS/AHAS inhibitors (Hall et al., 1999). The herbicides are applied directly to soil and weeds and, depending on the type of herbicide, may

remain in the soil until decomposed or, as in the case of glyphosate, may bind to soil particles and become inactive (Haney et al., 2000; Andréa et al., 2003).

Khan et al. (2004, 2006) explored the relationship between N_2 fixation and pre-emergent herbicide applications on chickpea. They performed a greenhouse study that included the pre-emergent herbicides bentazon, fluchloralin, isoproturon and 2,4-D applied at 1×, 2× and 10× the recommended field rates. Khan et al. (2004) examined the impact of herbicides on nodulation ability and rhizobial growth as an index for assessing the N_2-fixing efficiency of *Rhizobium* species in chickpea. At the 10× rate, all herbicides adversely affected plant vigor and N content in shoots and seeds. Additionally, all rates (except bentazon at 1× the recommended field rate) adversely affected rhizobial populations, both native and inoculated, within single nodules on the chickpea plant, as determined using an immunoblot assay. They acknowledged that a reduction in the photosynthate supply to the roots in the nodulating phase would result in a reduction in the number and/or size of nodules and suggested that a nutritional deficiency, rather than an herbicide toxicity, limited rhizobial cell growth in the nodules. They concluded that the herbicides affected nodule occupancy at higher dose rates by acting directly on the plant and only indirectly on the nodule bacteria. In a subsequent growth chamber experiment, Khan et al. (2006) used the pre-emergent herbicides, methabenzthiazuron, terbutryn and linuron, at 2.5, 1.25 and 2 g a.i. kg^{-1} soil in order to study the effects on N_2 fixation. There were no significant adverse effects of the herbicides when applied at recommended rates on plant growth, yield and NA of excised nodules in chickpea, but there were reductions when the herbicides were applied at higher than recommended rates. These studies indicate that different herbicides can have varying effects on N_2 fixation; thus, it is important to examine the impact of herbicides commonly used in western Canada with the goal of minimizing any negative effects and maximizing N_2 fixation efficacy.

Besides pre-emergent application, herbicides may also be applied as a post-emergent treatment, in which the crop plants are established and the herbicide applied is intended to kill target weeds but have little or no impact on the crop. These herbicides are classified by mode of action and include: cell membrane disruption [acetyl-CoA-carboxylase (ACCase) inhibitors that kill grasses but not broad-leaf plants]; amino acid

starvation (ALS inhibitors that are selective for either broad-leaf or grass); and disruption of amino acid synthesis (EPSPS inhibitors that kill all but glyphosate-tolerant plants) (Hall et al., 1999).

Rennie and Dubetz (1994) stated that post-emergent herbicides were not likely to affect nodulation, but Sawicka and Selwet (1998) and Gonzales et al. (1996) found otherwise. Under field conditions, Sawicka and Selwet (1998) applied imazethapyr as a post-emergent treatment to field pea, horse bean (*Vicia faba* L.), yellow lupine (*Lupinus luteus* L.) and soybean. They found that NA was reduced in all treated plants. Gonzales et al. (1996) also studied this relationship and found imazethapyr reduced nodulation by 45%. At doses higher than 1.73 μM g^{-1}soil, nodule formation was almost completely inhibited. Imazethapyr inhibits ALS activity, an enzyme that also occurs in rhizobia; therefore, it is possible that this herbicide may have a direct effect on microorganisms. Possible negative effects that post-emergent herbicides may have on N_2 fixation ought to be investigated.

In addition to herbicides applied either pre- or post-emergent to the N_2-fixing legume crop, there also is the possibility that herbicide residues from previous cropping seasons may affect the legume-*Rhizobium* association. In this case, the residual herbicide may not be registered for use in the legume crop the following year. For example, herbicides such as flucarbazone-sodium have entered the market and are known to have soil residual effects that may negatively affect legumes in subsequent years (Eliason et al.; 2004, Saskatchewan Agriculture and Food, 2005). Soil residues of some herbicides inhibit nodulation (Eberbach and Douglas, 1989; Mårtensson and Nilsson, 1989), N_2 fixation (Koopman et al., 1995) and may be phytotoxic (Eberbach and Douglas, 1983; Anderson, 1985). Eberbach and Douglas (1991) examined residual herbicide effects by applying amitrole and diquat at rates of 0, 2, 5 and 10 μg a.i. g^{-1} to a sandy loam soil and allowing it to degrade for 120 d. Amitrole applications at rates higher than 2 μg a.i. g^{-1} soil resulted in sufficient residues remaining to be detrimental to subterranean clover (*Trifolium subterraneum* L.) seedlings. Specifically, plant growth, nodulation and NA of plants were reduced. When 2,4-D was applied, plant growth and nodule formation were reduced, but NA was unaffected. While studies are very limited in Saskatchewan, results from other parts of the world are cause for concern as many

Saskatchewan producers use various soil residual Group 2 herbicides in their cropping systems and include legumes in their rotations.

Research in N_2 fixation is most applicable for growers when conducted at the field level under natural environmental conditions. This study was designed to answer the question: Will herbicide application in the field decrease the ability of rhizobia to fix atmospheric N_2? This study included two field experiments that were conducted to examine the impact of pre- and post-emergent herbicides on N_2 fixation parameters in field pea and chickpea. A third experiment included sowing field pea on soil that previously was treated with herbicides known to persist in soil the year following application. A split-plot design was used in the latter experiment to determine if granular (soil applied) or peat-based powder (seed applied) inoculants mitigated possible negative consequences that residual herbicides may have on N_2 fixation. Over the course of the experiments, N_2-fixation was assessed by using acetylene reduction assay (ARA, recorded as NA), and the amount of N derived from the atmosphere (Ndfa). Additionally, the relative effectiveness of the rhizobia was assessed by calculating the NA per unit nodule mass.

3.2 Materials and Methods

In 2005, two experiments were established near Clavet, Saskatchewan (SW-9-36-2, W3) and Floral, Saskatchewan (University of Saskatchewan Goodale Research Farm; SW-3-36-4, W3), to examine the impact of herbicide application in pulse crop production. Three experiments were conducted. The first experiment examined the impact of pre- and post-emergent herbicide application on N_2 fixation on field pea (Experiment 1). This experiment was conducted at both Clavet and Goodale. The second experiment, conducted only at Goodale, examined the impact of pre- and post-emergent herbicide application on chickpea (Experiment 2). The third field experiment took place over a two-year period in 2005 and 2006 at Goodale (Experiment 3) and examined the effects of residual herbicides previously applied to a wheat crop on N_2 fixation in field pea grown in the subsequent year. Additionally, the effectiveness of peat and granular inoculants on N_2 fixation, in response to residual herbicides, was assessed. Herbicide information including chemical name, trade name, timing of application, formulation, concentration and manufacturer is found in the Appendix

(Table A.1).

3.2.1 Site and experiment descriptions

Experiments 1 and 2 examined effects of pre- and post-emergent herbicides on N_2 fixation (Goodale and Clavet). They were organized according to a randomized complete block design (RCBD) with four replicates at Goodale and six at Clavet. Experiment 3 was designed to examine effects on N_2 fixation by residual herbicides, and used a split-plot design with main plots comparing herbicides and subplots comparing inoculation types (seed-applied peat-based powder or soil applied granular inoculants). There were four replicates. Site information including seeding rates, row spacing and dates for seeding, herbicide application and harvesting can be found in Table 3.1.

At Goodale, plots were 2.25 m wide and 6 m long. At Clavet, plots were 1.1 wide and 10 m long.

3.2.2 Soil analysis

Soil samples were collected in the fall preceding the growing season, using a hand-held Dutch auger, to a depth of 30 cm. Samples were gathered from five random locations in each replicate and were bulked. Subsamples were sent to EnviroTest Labs (ALS Laboratory Group, Saskatoon, SK). Analyses included pH and electrical conductivity (E.C.) [determined on a 1:2 soil water suspension (Hogg and Henry, 1984)], inorganic N (NO_3^-) [measured by KCl extraction (Maynard and Kalra, 1993)], phosphorus, as P_2O_5, and potassium as K_2O [measured using the modified Kelowna extraction method (Qian et al., 1994)]. Soil characteristics such as fertility (N, P and K) as well as E.C. and pH are described in Table 3.2.

3.2.3 Field assessment of the impact of pre- and post-emergent herbicide application on field pea (Experiment 1)

The experiment examined the impact of the pre-emergent herbicides amitrole and glyphosate, and the post-emergent herbicides MCPB + MCPA, MCPA, imazamox + imazethapyr, metribuzin and bentazon on N_2 fixation in field pea. The experiment included seven treatments and an untreated check and a non-N_2 fixing flax reference crop of flax (*Linum usitatissimum* L. cv. AC Watson) (Table 3.3). The herbicides used are registered for field pea (Saskatchewan Agriculture and Food, 2005 and 2006) and

Table 3.1 Site information for field trials at Goodale and Clavet, Saskatchewan.

Location and crop	Experiment	Seeding date		Seeding rate (depth)	Row spacing	Herbicide application date (pre/post)	Harvest	
		2005	2006			Day/month	2005	2006
				kg ha^{-1} (cm)	cm			
Goodale								
Field pea	Pre- and post–emergent (Experiment 1)	11/05	n/a	185 (4.5)	21.5	09/05 09/06	22/08	n/a
Chickpea	Pre- and post-emergent (Experiment 2)	05/05	n/a	66 (6)	21.5	09/05 09/06	12/10	n/a
Field pea	Residual (Experiment 3)	11/05	11/05	185 (4.5)	21.5	15/06/2004 14/06/2005	22/08	04/08
Flax†	Experiments 1, 2 and 3	12/05	11/05	35(2.5)	21.5	n/a	22/08	21/08
Clavet								
Field pea	Pre- and post-emergent (Experiment 1)	14/05	n/a	185(2.5)	20.3	19/05 09/06	19/08	n/a
Flax†		14/05	n/a	35(2.5)	20.3	n/a	08/09	n/a

† Reference crop used to determine N_2 fixation.

Table 3.2 Soil descriptions for Clavet and Goodale field sites†.

	2005			2006
Location	Clavet (Experiment 1)	Goodale In-crop (Experiment 1 and 2)	Goodale Residual (Experiment 3)	Goodale Residual (Experiment 3)
Soil Association	Elstow	Bradwell	Bradwell	Bradwell
Soil order	Orthic Dk Brown	Orthic Dk Brown	Orthic Dk Brown	Orthic Dk Brown
Soil texture	Clay loam	Fine sandy loam	Fine sandy loam	Fine sandy loam
pH	7.3	7.0	7.0	7.2
E.C. (mS cm^{-1})	1.1	0.7	0.7	0.7
Soil test N (μg g^{-1})	27	14	14	9
Soil test P (μg g^{-1})	27	>60	>60	>60
Soil test K (μg g^{-1})	>600	>600	>600	>600

†Analyzed samples were collected to a depth of 30 cm.

Table 3.3 Pre- and post-emergent herbicide treatment descriptions for field pea experiments in Clavet and Goodale (Experiment 1).

Herbicide (adjuvant)	Mode of Action	Rate (g a.i. ha^{-1})	Timing‡ (Crop Stage)
Amitrole	Inhibits carotenoid synthesis (Group 11)	970	Pre-emerg.
Glyphosate	Inhibits EPSP synthase (Group 9)	450	Pre-emerg.
MCPB + MCPA	Synthetic auxin (Group 4)	1594+106	Post-emerg. (3-6 leaf)
MCPA	Synthetic auxin (Group 4)	270	Post-emerg. (10-18 cm height)
Imazethapyr + imazamox (Merge)	ALS/AHAS inhibitors (Group 2)	15+15 0.5†	Post-emerg. (1-6 node)
Metribuzin	Photosynthetic inhibitor (Group 5)	280	Post-emerg. (up to 15 cm height)
Bentazon (Cittowet)	Photosynthetic inhibitor (Group 6)	1080 0.25†	Post-emerg. (after 3 pairs of leaves)
Untreated check	-	-	
Flax	-	-	

† volume:volume.

‡ Label recommended timing of application.

were applied at recommended field rates. Treatments, rates, mode of action and timing are described in Table 3.3.

At Goodale, pre- and post-emergent herbicides were applied using a tractor mounted plot sprayer. The sprayer used at Goodale was equipped with Greenleaf Air Mix Am110015® nozzles on a 1.8 m wide boom set at a height of 0.46 m above the crop canopy. Travel speed was 7.2 km h^{-1}. At Clavet, pre- and post-emergent herbicides were applied with a 2 m hand-held sprayer with flat fan XR 80015® nozzles. Nozzles were spaced 0.5 m apart and the boom was held at a height of approximately 0.46 m above the canopy at a walking speed of approximately 7.2 km h^{-1}.

Field pea (var. CDC Mozart) seed was inoculated with a peat-based inoculant [NitraStiK C® provided by Nitragin (EMD Crop BioScience**,** Brookfied, IL)] that was applied at a rate of 4 g kg^{-1} of seed.

3.2.4 Field assessment of the impact of pre- and post-emergent herbicides on chickpea (Experiment 2)

The impact of pre- and post-emergent herbicides on N_2 fixation in chickpea (cv. CDC Desiray) was examined using a single experiment conducted at the Goodale site. Experimental design was a RCBD with five herbicide treatments and four replicates. The herbicides used in this study included the only registered herbicide (metribuzin) applied at the recommended field rate and twice the recommended field rate (Table 3.4). All other treatments, e.g., sulfentrazone, isoxaflutole and, imazethapyr + glyphosate, were not registered for use in chickpea and were included in this trial because they were being considered for minor use registration when the experiment was initiated. Herbicide description and application rates and timing are given in Table 3.4. Flax was the non-N_2-fixing reference crop.

Chickpea was inoculated with Soil Implant® (provided by Nitragin) applied at a rate of 6.8 kg ha^{-1}. Pre- and post-emergent herbicides were applied with equipment and methodology as described previously for the Goodale site (Section 3.2.3).

Table 3.4 Herbicide treatments used in the chickpea field experiment at Goodale in 2005 (Experiment 2).

Chemical Name	Mode of Action	Rate (g a.i. ha^{-1})	Timing (Crop stage)
Metribuzin	Photosynthetic inhibitor (Group 5)	205	Post-emerg. (1-3 nodes)
Metribuzin	Photosynthetic inhibitor (Group 5)	410	Post-emerg. (1-3 nodes)
Sulfentrazone	Photosynthetic inhibitor (Group 14)	280	Pre-emerg.
Isoxaflutole	Photosynthetic inhibitor (Group 28)	105	Pre-emerg.
Imazethapyr + glyphosate	ALS/AHAS inhibitor, inhibits EPSP synthase (Groups 2,9)	16.5+450	Pre-emerg.
Untreated check	-	-	-
Flax	-	-	-

3.2.5 Field assessment of the impact of residual herbicides on field pea (Experiment 3)

Herbicides including flucarbazone-sodium, clopyralid and sulfosulfuron are known to have residual effects in subsequent years (Saskatchewan Agriculture and Food, 2006). Experiment 3 examined the impact of these residual herbicides (applied to a previous wheat crop) on N_2 fixation in field pea. This experiment was conducted over two growing seasons at the Goodale site. Wheat was treated with the herbicides in year 1, grown to maturity, harvested using a small-plot combine and crop residues were left on the soil surface. The subsequent pea crop was seeded directly into the stubble of the previous wheat crop. The experiment was conducted using a split-plot design with four herbicide treatments as the main-plot and inoculant type (peat versus granular) as the sub-plot. The treatments were replicated four times and included two hand-weeded untreated checks and two flax reference treatments per replicate.

Field pea (cv. CDC Mozart) was inoculated with either a peat-based or granular-based inoculant. The peat-based inoculant was NitraStiK C® while the granular inoculant used was Soil Implant®, both provided by Nitragin. Inoculants were applied at recommended rates of 4 g kg^{-1} seed and 5.6 kg ha^{-1}, respectively.

Herbicides in this experiment were registered products and were applied at recommended field rates (Saskatchewan Agriculture and Food, 2005; 2006). In the case of flucarbazone-sodium, two rates were used; the field recommended 20 g a.i. ha^{-1} (for broadleaf or light infestations of foxtail) and 30 g a.i. ha^{-1} (for heavy infestations of foxtail). Descriptions of the herbicide treatments (mode of action, rate and timing) are found in Table 3.5.

Table 3.5 Treatment description for residual herbicide (Experiment 3) at Goodale in 2005 and 2006.

Herbicide (adjuvant)	Mode of action	Herbicide rate (g a.i. ha^{-1})	Timing‡ (crop stage)	Inoculant
Clopyralid + MCPA, clodinafop-propargyl (Score)	Inhibits growth, ACCase (Groups 4,1)	660+56 (0.8†)	Post-emerg. (3 leaf)	Peat
Clopyralid + MCPA, clodinafop-propargyl (Score)	Inhibits growth, ACCase (Groups 4,1)	660+56 (0.8†)	Post-emerg. (3 leaf)	Granular
Flucarbazone-sodium (Agral 90)	ALS/AHAS inhibitor (Group 2)	20 (0.25†)	Post-emerg. (1-4 leaf)	Peat
Flucarbazone-sodium (Agral 90)	ALS/AHAS inhibitor (Group 2)	20 (0.25†)	Post-emerg. (1-4 leaf)	Granular
Flucarbazone-sodium (Agral 90)	ALS/AHAS inhibitor (Group 2)	30 (0.25†)	Post-emerg. (1-4 leaf)	Peat
Flucarbazone-sodium (Agral 90)	ALS/AHAS inhibitor (Group 2)	30 (0.25†)	Post-emerg. (1-4 leaf)	Granular
Sulfosulfuron (Finnish + Merge)	ALS/AHAS inhibitor (Group 2)	20 (0.25+0.5†)	Post-emerg. (1-4 leaf)	Peat
Sulfosulfuron (Finnish + Merge)	ALS/AHAS inhibitor (Group 2)	20 (0.25+0.5†)	Post-emerg. (1-4 leaf)	Granular
Untreated check				Peat
Untreated check				Granular
Flax				
Flax				

† volume:volume (%) surfactant.
‡ Label recommended timing of application.

3.2.6 Sampling and Analysis

3.2.6.1 Crop injury

In 2005 and 2006 visual assessments of crop injury [Expert Committee on Weeds (ECW) rating scale (1988) subsequently adopted by the Canadian Weed Science Society] were conducted on all field trials. Visual assessments define phytotoxicity as described in Table 3.6. At Goodale, visual assessments for Experiment 1 (pre- and post-emergent herbicide application on field pea) and Experiment 2 (pre- and post-emergent herbicide application on chickpea) were recorded 7 and 35 d after the post-emergent herbicide application. In 2005, one visual assessment was conducted during flowering for Experiment 3 (residual herbicide on field pea), and was carried out at the same time as Experiment 1 (more than one year after herbicide application). In this year, there were no further assessments for Experiment 3 as there were no visible differences detected during the first assessment and it was assumed that no further damage would be observed. Similarly, in 2006, there was only one visual assessment for Experiment 3 as there were no visible differences between treatments. In 2005, at Clavet (Experiment 1), visual assessments took place 7 and 29 d after application (DAA) of the post-emergent herbicides.

3.2.6.2 Biomass

At the same time injury assessments were performed, ARAs and above-ground biomass measurements were completed. Within each replicate and in each treatment, three plants were randomly chosen from within the plot, and the plant, including the roots, was excavated using a shovel to a depth of approximately 20 cm. The above-ground biomass was separated from the roots, and both were placed in paper bags to be subsequently dried for 72 h at 45˚C and weighed. Usually biomass sampling assessments are based on dry matter collected from a defined area (i.e., 1 m^2); however, for these experiments, biomass collected for the ARA assessment was dried and recorded to examine the relationship between biomass and N_2-fixing parameters.

Table 3.6 Crop tolerance as described by the Expert Committee on Weeds (1998).

Phytotoxicity	Injury
(%)	
0-9	Slight discoloration/stunting
10	Just acceptable
11-30	Not acceptable
>30	Severe

Biomass measurements were not intended to be indicative of the entire field. The roots were immediately used for assessment of N_2-fixing activity using the ARA. Following ARA analyses (recorded as NA) in the field, root samples were taken to the lab and washed with tap water. Nodules were removed using a scalpel and placed in pre-weighed aluminum foil weigh boats. Nodule fresh weight was recorded and then the nodules were oven-dried at 60˚C for 24 h and weighed.

3.2.6.3 Acetylene reduction assays

The ARA method was used to measure the N_2-fixing activity of the nodules (Hardy et al., 1973). Three plants were removed from the soil, as described above, and shaken gently. Shoots were cut off and the exposed roots were placed in a 1 L Mason jar. One hundred milliliters of headspace was removed using a 40 mL syringe and replaced with 100 mL of acetylene (C_2H_2). Jars were buried in loose soil up to the lids and intermittently shaken in place to maximize nodule exposure to acetylene. Before sampling, the air in the jars was mixed by pumping the syringe four to five times to ensure a homogeneous mixture of the gases. Following incubation for 30 min, 10 mL (2005) or 20 mL of gas (2006) were removed and placed into 12 mL evacuated vacutainers (Labco Ltd., Buckinghamshire, U.K.). Samples were analyzed using a gas chromatograph (GC; Hewlett-Packard 5890A) fitted with a flame ionization detector (FID) for the concentration of nitrogenase-reduced acetylene. Chromotographic separation was carried out on a 1.8m Porplak™Q column (80/100 mesh), and the

instrument was run at 60°C with N as the carrier gas at a flow rate of 40 cc min^{-1}.

3.2.6.4 Yield determination and nitrogen derived from the atmosphere

Four crop rows, 1 m long were cut at the soil surface using a hand-held sickle. Samples were placed in cloth bags and hung to air dry. These samples were used to determine yield, seed N, total N, N derived from the atmosphere (Ndfa), protein content and harvest index (i.e., the ratio of seed biomass to total above-ground biomass). Air-dried shoot biomass was measured, and samples threshed and seed collected. Seed was dried at 60°C for 1 wk, weighed and ground to pass a 2 mm sieve. Ground samples were further pulverized to a fine powder in a ball mill. Subsamples (1±0.05 mg) were analyzed for ^{15}N natural abundance using an ANCA elemental analyzer coupled to a TraceMass mass spectrometer (Europa Scientific, Crewe, U.K.) at the Stable Isotope Facilities at the Department of Soil Science, University of Saskatchewan. Finely ground field pea seed with an atom % ^{15}N of 0.3675 was used as a working standard (Bremer and van Kessel, 1990). Percent N derived from the atmosphere (%Ndfa) was calculated using the following equation:

$$\%Ndfa = \left[\frac{(x-y)}{(x-c)} \right] 100 \qquad \text{Eq [3.1]}$$

Where x = $\delta^{15}N$ of seeds deriving all their N from the soil (in this case, flax), y = $\delta^{15}N$ of the seeds of field pea or chickpea. The value for c represents $\delta^{15}N$ value of pea or chickpea grown in an N-free medium. The c value used for field pea was 0.7 (Bremer and van Kessel, 1990) and for chickpea was 1.0005 (Kyei-Boahen, 2000). All negative estimates of %Ndfa are reported as zero (Bremer et al., 1988).

3.2.6.5 Nitrogen accumulation

Nitrogen accumulation in the plant tissue (percent N) was determined using a LECO CNS-2000. The amount of N accumulated in the plant was determined by multiplying percent N and shoot biomass. Protein content was calculated using percent N and multiplying by a conversion factor of 6.25 (Sosulski and Imafidon, 1990).

3.2.6.6 Environmental conditions

Temperature and precipitation were measured at the Goodale site with a WatchDog Model 2550 weather station (Spectrum Technologies, 2008). The weather station measures real-time wind speed and direction, temperature and relative humidity as well as rainfall (tipping bucket).

3.2.6.7 Statistical analysis

The data were analyzed using SPSS 14.0 for Windows (SPSS 14.0, 2005) to determine normality. In cases where data were not normal, they were transformed using the log function (Bland and Altman, 1996). Data were examined using the General Linear Model and analysis of variance (ANOVA) (SAS, 1996). In Experiments 1 and 2, differences between the treatments and the control were determined using the Dunnett test at $P \leq 0.05$. In Experiment 3, least significant differences (LSD) were determined using repeated-measures split-plot ANOVA (SAS, 1996). Rankings were unchanged by transformations; therefore, statistical analyses are reported for untransformed data.

3.3 Results

3.3.1 Environmental conditions

Weather data collected on-site (pers.comm, Gerry Stuber, University of Saskatchewan) for 2004, 2005 and 2006 are summarized in Table 3.7. In 2004, precipitation in June, July and August was higher than the 10 yr normal (1990-2000) as reported by Environment Canada (2000). Temperature ranged below the average from -0.7 to -3.6 ºC. In 2005, precipitation again was higher than normal for June, August and September. Ninety-three percent of normal precipitation fell in July. Again, temperatures were slightly below the 10 yr average and ranged from -0.6 to -2.1 ºC below normal. Weather data in 2006 indicated higher than normal precipitation in May and June and only slightly below average precipitation in July and August (76% and 91%, respectively, of normal), relative to the 10 yr average.

Table 3.7 Temperature averages and precipitation for 2004, 2005 and 2006.

Month	Temperature				Precipitation			
	2004	2005	2006	10 yr average	2004	2005	2006	10 yr average
		ºC				mm		
May	7.9	9.8	11.0	11.5	33.7	28	58.2	49.4
June	13	14.6	16.2	16	94.5	173	110.8	61.1
July	17.1	18.0	20.0	18.2	80.6	57	45.8	60.1
August	13.9	15.3	18.1	17.3	76.6	84	35.4	38.8
September	10.5	11.4	12.0	1.2	17.1	93	125.2	30.7
October	2.8	5.1	1.5	4.5	28.9	13	39.0	16.7

3.3.1 Field assessment of the impact of pre- and post-emergent herbicide application on field pea (Experiment 1)

Experiment 1 examined the impact of pre- and post-emergent herbicide application on N_2 fixation in field pea. Pre-emergent herbicides included amitrole and glyphosate, and post-emergent herbicides included MCPB + MCPA, MCPA, imazamox + imazethapyr, metribuzin and bentazon. Results from the ANOVA are presented in the appendix (Table A.2; Table A.3; Table A.4; Table A.5; Table A.6; Table A.7)

3.3.1.1 Visual assessments of crop injury

Experiment 1 was conducted at both Goodale and Clavet in 2005. Field pea grown at Goodale initially showed minimal visual differences among treatments 7 DAA (Note: DAA always refers to days after the post-emergent herbicide application) (Table 3.8) and all treatment injury was considered acceptable, according to the guidelines of the ECW (1988) (ratings of 2 and 3 are so low as to likely have no biological effect). Thirty-five days after post-emergent herbicide application, treatments including MCPB + MCPA and MCPA increased plant injury (Table 3.8). According to the ECW (1988), the phytotoxicity to the field pea with these two herbicide applications is not acceptable suggesting that there may be a loss in yield or protein at harvest.

Table 3.8 Visual assessments of injury to field pea at Goodale and Clavet in 2005 (Experiment 1) based on the Expert Committee on Weeds rating system (1988).

Treatment	Rate	Plant Injury			
	(g a.i. ha^{-1})	Goodale		Clavet	
		Day/month		Day/month	
		16/06 (7 DAA)	07/07 (35 DAA)	16/06 (7 DAA)	07/07 (29 DAA)
Amitrole	970	2	1	2	0
Glyphosate	450	0	0	2	0
MCPB + MCPA	1594 +106	0	11	2	9
MCPA	15 + 15	0	12	1	6
Imazamox + imazethapyr	30	4	5	3	3
Metribuzin	280	3	5	1	3
Bentazon	1080	4	8	3	6
Untreated check		0	0	0	0

Field pea grown at Clavet displayed little visual damage 7 DAA and all plots were considered to have acceptable damage. Twenty-nine days after application, pea treated with MCPB + MCPA, MCPA and bentazon were slightly damaged, but were still considered to have an acceptable amount of injury that normally would be expected to have little negative consequence on yield (Table 3.8).

3.3.1.2 Impact of herbicide application on early season nitrogen fixation and biomass

Seven DAA, shoot biomass, nodule weight, NA and N accumulation in field pea grown at Clavet were all reduced by herbicide treatments relative to the control (Table 3.9). For example, relative to the untreated control, shoot biomass was reduced ($P \leq 0.05$) by glyphosate, MCPB + MCPA, MCPA, and bentazon. Similarly, N accumulation was reduced by glyphosate and MCPB + MCPA.

Nitrogenase activity was significantly reduced by treatment with MCPB + MCPA. In order to assess the efficacy of the rhizobia in the nodules, NA per unit nodule weight (termed specific NA) was assessed. While NA was reduced by MCPB + MCPA, the efficacy of the rhizobia was unaffected; however, specific NA increased by amitrole and bentazon treatments as compared to the control.

In the same sampling period, nodule biomass was significantly lower than the control for pea in plots treated with amitrole, MCPB + MCPA, MCPA and metribuzin (Table 3.9).

Differences detected 7DAA for these measurements were not detected 35 DAA (Table 3.9). All measurements recorded 35 DAA were not significant according to the ANOVA ($P \leq 0.05$) (Table 3.9; Table A.2; Table A.3)

There were no detectable differences between the different herbicide treatments and the untreated control for shoot biomass, NA, and nodule biomass both 7 DAA and 35 DAA sampling periods at Goodale (Table 3.10; Table A.5; Table A.6). The only measurement that was different between treatments and the control was nodule efficacy in June for MCPB + MCPA and MCPA (Table 3.10). Thirty five days after application, these differences were no longer detectable.

Table 3.9 The impact of pre- and post-emergent herbicides on early season biomass and N_2 fixation parameters of field pea at Clavet, SK (2005).

Treatment	Rate	Shoot Biomass†		N accumulation		NA‡		Nodule biomass		Specific NA	
		Days after application									
		7	29	7	29	7	29	7	29	7	29
	g a.i. ha^{-1}	g		mg		µmol ethylene h^{-1}		mg		µmol ethylene g^{-1} nodule h^{-1}	
Amitrole	970	1.39	5.51	6.6	19.8	148	1182	19.3*	462.6	7276*	2718
Glyphosate	450	1.26*	5.62	5.6*	21.2	154	1314	25.4	520.1	6198	2738
MCPB + MCPA	1594 + 106	1.11*	5.54	5.1*	22.2	74*	904	18.4*	308.5	4288	3048
MCPA	270	1.22*	5.73	5.9	22.9	108	1358	18.7*	481.6	5740	2790
Imazethapyr + imazamox	15 + 15	1.44	6.19	6.9	23.4	152	854	24.6	315.7	6350	2652
Metribuzin	280	1.35	5.33	6.5	21.6	116	772	21.3*	297.8	6050	2492
Bentazon	1080	1.25*	5.23	5.6*	20.7	166	912	23.9	318.8	7470*	2986
Untreated check	-	1.52	6.41	6.9	23.1	170	906	34.2	432.0	4904	2604

* denotes significantly different than control at $P \leq 0.05$ using Dunnett's test to compare means.
† data based on three plants per replicate.
‡ NA = nitrogenase activity.

Table 3.10 The impact of pre- and post-emergent herbicides on early season biomass and N_2 fixation parameters of field pea at Goodale (2005).

Treatment	Rate	Shoot Biomass†		N accumulation		NA‡		Nodule biomass		Specific NA	
		Days after application									
		7	35	7	35	7	35	7	35	7	35
	g a.i. ha^{-1}	g		mg		µmol ethylene h^{-1}		mg		µmol ethylene g^{-1} nodule h^{-1}	
Amitrole	970	2.36	12.49	9.5	39.2	1034	2876	184.3	837.7	5478	3554
Glyphosate	450	2.14	12.73	8.3	37.0	856	2456	143.2	651.4	5964	3878
MCPB + MCPA	1594 + 106	1.51	8.18	6.6	47.4	456	1740	151.0	617.8	3016*	2982
MCPA	270	1.88	9.04	7.7	40.3	758	2144	198.4	712.2	3782*	3148
Imazethapyr + imazamox	15 + 15	1.93	9.09	7.4	65.7	746	2322	144.9	673.7	5238	3530
Metribuzin	280	2.20	13.67	9.5	37.1	822	2120	161.4	805.0	5156	2800
Bentazon	1080	1.49	8.28	6.4	47.0	536	2476	94.2	647.8	5608	3966
Untreated check		2.18	12.98	8.2	34.3	840	2822	158.6	962.5	5476	3156

* denotes significantly different than control at $P \leq 0.05$ using Dunnett's test to compare means.
† data based on three plants per replicate.
‡ NA = nitrogenase activity.

3.3.1.3 Impact of herbicide application on nitrogen fixation, yield and harvest index at final harvest

There were no observable reductions in N_2 fixation in field pea grown at Clavet and Goodale including N accumulation (Table 3.9; Table 3.10), protein content, %Ndfa, and Ndfa in seed (kg ha^{-1}) (Table 3.11; Table 3.12 . Yield and harvest index did not differ from the control at both Clavet and Goodale (Table 3.11; Table A.4; Table A.7).

3.3.2 Field assessment of the impact of pre- and post-emergent herbicides on chickpea (Experiment 2)

Experiment 2 examined the impact of pre- and post-emergent herbicide application on N_2 fixation in chickpea. Herbicides included pre-emergent application of sulfentrazone, isoxaflutole and imazethapyr + glyphosate. Metribuzin was applied as a post-emergent treatment at the recommended field rate and at twice the recommended field rate. Results from the ANOVA are presented in the appendix (Table A.8; Table A.9; Table A.10).

3.3.2.1 Visual assessments of crop injury

When assessed seven days after post-emergent herbicide application, chickpea treated with metribuzin at both the recommended and twice the recommended field rates were visibly damaged and the degree of injury was considered unacceptable according to ECW guidelines (1988) (Table 3.13 and Fig 3.1). Application of metribuzin typically resulted in significant losses of exposed leaves, and reduced branching and height (Fig. 3.1). No visual damage was detected from sulfentrazone, isoxaflutole and imazethapyr + glyphosate applied as pre-emergent treatments. In the second sampling (35 DAA), chickpea plants receiving metribuzin at the recommended field rate recovered and any visual damage detected was considered acceptable; however chickpea treated with metribuzin at the 2× rate partially recovered, but not enough for the injury to be considered acceptable according to the ECW (1988). Chickpea in other treatments (sulfentrazone, isoxaflutole and imazethapyr + glyphosate) exhibited more visual damage as compared to the earlier assessment; however, the damage was acceptable and not likely to have any significant effect on yield.

Table 3.11 The impact of pre- and post-emergent herbicides on yield and N_2 fixation determined at final harvest of field pea at Clavet (2005).

Treatment	Rate	Seed Yield	Seed Protein	Ndfa	Seed Ndfa	Harvest Index
	g a.i. ha^{-1}	kg ha^{-1}	%	%	kg ha^{-1}	
Amitrole	970	3130	22.4	17	20	0.53
Glyphosate	450	3400	22.0	13	16	0.53
MCPB + MCPA	1594 + 106	3170	22.0	13	16	0.51
MCPA	270	2840	22.6	8	7	0.51
Imazethapyr + imazamox	15 + 15	3600	22.3	1	0	0.52
Metribuzin	280	3180	21.6	6	6	0.51
Bentazon	1080	3080	21.3	1	2	0.51
Untreated check		2970	22.6	3	2	0.51

Table 3.12 The impact of pre- and post-emergent herbicide application on yield and N_2 fixation determined at final harvest of field pea at Goodale (2005).

Treatment	Rate	Seed Yield	Seed Protein	Ndfa	Seed Ndfa	Harvest Index
	g a.i. ha^{-1}	kg ha^{-1}	%	%	kg ha^{-1}	
Amitrole	970	4460	20.6	45	73	0.56
Glyphosate	450	4580	21.5	59	99	0.55
MCPB + MCPA	1594 + 106	4510	20.5	47	86	0.56
MCPA	270	3990	21.2	77	107	0.55
Imazethapyr + imazamox	15 + 15	4900	21.0	67	120	0.57
Metribuzin	280	3430	22.0	70	72	0.44
Bentazon	1080	4170	21.3	49	77	0.58
Untreated check		4350	21.8	49	78	0.56

Table 3.13 Visual assessments of herbicide injury according to ECW guidelines (1988) for chickpea grown at Goodale, SK.

Treatment	Rate	Plant Injury	
		Day/month	
	g a.i. ha^{-1}	16/06 (7 DAA)	07/07 (35 DAA)
Metribuzin	205	43	9
Metribuzin	410	43	18
Sulfentrazone	280	0	4
Isoxaflutole	105	0	5
Imazethapyr + glyphosate	16.5 + 450	0	4
Untreated check	n/a	0	0

Figure 3.1 Comparison between metribuzin (2× rate) treated chickpea plant (left) and an untreated plant (right). Plants were collected from the field trial at Goodale, SK.

3.3.2.2 Impact of herbicide application on early season nitrogen fixation and biomass

At the first sampling period (7 DAA), shoot biomass was significantly lower in chickpea treated with metribuzin at both 1× and 2× rates when compared to the control (Table 3.14). Nodule biomass was significantly reduced by the application of metribuzin at the 2× rate as compared to the control (Table 3.14; Table A.8). During this sampling period, there were no differences in NA between the herbicide treatments and the control, and the efficacy of the rhizobia (i.e., specific NA) also remained unaffected (Table 3.14; Table A.9). At the subsequent sampling date in July (35 DAA), shoot biomass recovered in both metribuzin treatments, however nodule biomass did not recover. Metribuzin applied at both the recommended rate and at the 2× rate reduced nodule biomass compared to the control. Although no detectable differences in NA were observed 7 DAA, NA in treatments including metribuzin applied at both rates and sulfentrazone was reduced relative to the control when assessed 35 DAA. Metribuzin application (2× rate), resulted in a 55% reduction in NA, followed by the metribuzin treatment at the recommended rate (1×) with a reduction of 52% and sulfentrazone (48%). When assessing the efficacy of the nodules (specific NA), there were no detected differences at either the June or July sampling periods (Table 3.14; Table A.9).

3.3.2.3 Impact of herbicide application on nitrogen fixation, yield and harvest index at final harvest of chickpea

There were no observable differences in overall N_2 fixation parameters between herbicide treatments and the control. Although there was a 6-fold difference in %Ndfa between amitrole and the control and a 10-fold difference in seed Ndfa, differences were not statistically significant due to the large variability in the data. Nitrogen derived from the atmosphere, seed Ndfa, protein content, yield and harvest index were unaffected by the herbicide treatments (Table 3.15; Table A.10).

Table 3.14 The impact of pre- and post-emergent herbicides on early season biomass and N_2 fixation parameters of chickpea at Goodale, SK (2005).

Treatment	Rate	Shoot Biomass†		N accumulation		NA‡		Nodule biomass		Specific NA	
		Days After Application									
		7	35	7	35	7	35	7	35	7	35
	g a.i. ha^{-1}	g		mg		µmol ethylene h^{-1}		mg		µmol ethylene g^{-1} nodule h^{-1}	
Metribuzin	205	0.94*	3.57	2.9	9.4	41	227*	29.3	202.2*	2438	2316
Metribuzin	410	1.00*	2.73	3.5	7.6	13	212*	14.2*	177.1*	1586	2414
Sulfentrazone	280	1.76	5.32	4.6	12.2	23	246*	48.3	314.6	752	1688
Isoxaflutole	105	1.48	5.31	4.1	14.8	43	400	44.4	395.2	1950	2124
Imazethapyr + glyphosate	16.5 + 450	1.63	5.76	4.4	14.0	27	310	47.1	337.1	1136	1808
Untreated check		1.51	4.00	4.0	10.4	33	475	46.4	390.8	1662	2448

***** denotes significantly different than control at $P \leq 0.05$ using Dunnett's test to compare means.
† data based on three plants per replicate.
‡ NA = nitrogenase activity.

Table 3.15 The impact of pre- and post-emergent herbicides on early season harvest yield and N_2 fixation parameters of chickpea (2005).

Treatment	Rate	Seed Yield	Seed Protein	Ndfa	Seed Ndfa	Harvest Index
	g a.i. ha^{-1}	kg ha^{-1}	%	%	kg ha^{-1}	
Metribuzin	205	1900	22.0	51	31	0.24
Metribuzin	410	1580	20.4	38	22	0.24
Sulfentrazone	280	2620	21.9	51	47	0.30
Isoxaflutole	105	2190	21.5	58	25	0.24
Imazethapyr + glyphosate	16.5 + 450	2720	22.5	44	41	0.35
Untreated check		2420	23.5	31	18	0.28

3.3.3 Field assessment of the impact of soil residual herbicides on field pea (Experiment 3).

Experiment 3 examined the impact of soil residual herbicides on N_2 fixation in field pea. Two types of inoculants were used in this experiment – peat-based powder (seed applied) and granular (soil applied). The inoculant formulation treatment was included to determine if inoculant formulation would mitigate any potential negative effects of the herbicide treatments. Wheat was planted the previous year (2004) and was sprayed with flucarbazone-sodium (20 and 30 g a.i.), clopyralid + MCPA and clodinafop-propargyl as well as sulfentrazone.

In 2005, there were no differences between the treatments and control detected in early season measurements including shoot biomass, N accumulation, NA and nodule biomass and specific NA (Table 3.16; Table A.11). In post-harvest measurements in 2005, % Ndfa was significantly lower than the control in pea planted in residual treatments including clopyralid + MCPA and clodinafop propargyl, flucarbazone (30 g a.i.) and sulfosulfuron. However, seed Ndfa (kg ha^{-1}) was unaffected by herbicide treatments (Table 3.17; Table A.12).

Comparing peat inoculated and granular inoculated field pea, peat inoculation increased NA and specific NA but no post harvest measurements including yield, protein, Ndfa, seed Ndfa and harvest index (Table 3.16; Table 3.17; Table A. 11; Table A.12). Shoot biomass was the only parameter affected by a significant interaction detected between the inoculant and the herbicide treatments. When comparing plants inoculated with peat-based powder versus the granular formation, plants inoculated with the granular formulation had increased shoot biomass in clopyralid + MCPA, and flucarbazone-sodium (20 g a.i. and 30 g a.i.) treatments whereas plants inoculated with the peat-based powder formulation sown in sulfosulfuron and in the untreated check had an increase in shoot biomass.

In 2006, no treatment differences were observed in any of the measurements (Table 3.18; Table A. 13). There were no differences when comparing inoculant types (Table 3.19; Table A. 14).

Table 3.16 The impact of residual herbicides and inoculant type on early season biomass and N_2 fixation parameters of field pea at Goodale (2005).

Treatment	Rate	Inoculant	Shoot Biomass†	N accumulation	NA‡	Nodule biomass	Specific NA
	g a.i. ha^{-1}		g	mg	µmol ethylene h^{-1}	mg	µmol ethylene g^{-1} nodule h^{-1}
				Herbicide × inoculant means			
Clopyralid + MCPA and clodinafop propargyl	660, 56	Peat	2.05	9.0	724	106.0	6582
		Granular	2.17	10.4	486	73.6	6528
Flucarbazone-sodium	20	Peat	1.80	8.0	826	135.7	6108
		Granular	2.10	9.7	672	153.7	4682
Flucarbazone-sodium	30	Peat	1.81	7.9	662	148.0	6040
		Granular	2.24	9.9	672	140.0	4886
Sulfosulfuron	20	Peat	2.23	9.1	758	148.2	5250
		Granular	2.00	8.6	678	145.2	4964
Untreated check		Peat	2.14	8.6	832	159.1	5432
		Granular	1.91	8.0	670	134.1	1014
					Herbicide means		
Clopyralid + MCPA and clodinafop propargyl	660, 56		2.11	9.7	606	89.3	6554
Flucarbazone-sodium	20		1.95	8.8	750	144.7	5396
Flucarbazone-sodium	30		2.02	8.9	776	144.0	5464
Sulfosulfuron	20		2.11	8.9	718	146.7	5108
Untreated check			2.02	8.3	752	146.6	5182
$LSD_{0.05}$			NS§	NS	NS	NS	NS
					Inoculant means		
		Peat	2.09	8.5	804a*	139.3	5884a
		Granular	2.00	9.3	634b	129.1	5198b
$LSD_{0.05}$			NS	NS	62	NS	214

* Means followed by the same letter within each column are not different according to the least significant difference ($P \leq 0.05$).
† data based on three plants per replicate.
‡ NA = nitrogenase activity.
§ NS = not significantly different $P \leq 0.05$.

Table 3.17 The impact of residual herbicides and inoculant type on final harvest seed yield and N_2 fixation parameters of field pea at Goodale (2005).

Treatment	Rate	Inoculant	Seed Yield	Seed Protein	Ndfa	Seed Ndfa	Harvest index
	g a.i. ha^{-1}		kg ha^{-1}	%	%	kg ha^{-1}	
				Herbicide × inoculant means			
Clopyralid + MCPA and clodinafop propargyl	660, 56	Peat	5060	22.0	65	118	0.5
		Granular	5500	21.5	67	126	0.49
Flucarbazone-sodium	20	Peat	6540	22.4	71	147	0.62
		Granular	6460	21.4	73	170	0.56
Flucarbazone-sodium	30	Peat	6000	22.4	70	166	0.53
		Granular	6780	22.1	68	148	0.65
Sulfosulfuron	20	Peat	5840	21.9	65	141	0.43
		Granular	6170	21.1	73	144	0.50
Untreated check		Peat	6870	21.8	84	170	0.52
		Granular	5820	21.4	76	188	0.50
				Herbicide means			
Clopyralid + MCPA and clodinafop propargyl	660, 56		5280	21.8	66b	122	0.50
Flucarbazone-sodium	20		6500	21.9	72ab	167	0.59
Flucarbazone-sodium	30		6390	22.2	69b	159	0.59
Sulfosulfuron	20		6000	21.5	69b	142	0.47
Untreated check			6350	21.6	80a	175	0.56
$LSD_{0.05}$			NS	NS	9	NS	NS
				Inoculant means			
		Peat	6060	22.1	71	154	0.52
		Granular	6150	21.5	71	152	0.54
$LSD_{0.05}$			NS	NS	NS	NS	NS

† NS = not significantly different $P \leq 0.05$.

Table 3.18 The impact of residual herbicides and inoculant type on early season biomass and N_2 fixation parameters of field pea at Goodale (2006).

Treatment	Rate	Inoculant	Shoot Biomass†	N accumulation	NA‡	Nodule biomass	Specific NA
	g a.i. ha^{-1}		g	mg	µmol ethylene h^{-1}	mg	µmol ethylene g^{-1} nodule h^{-1}
					Herbicide × inoculant means		
Clopyralid + MCPA and clodinafop propargyl	660, 56	Peat	11.5	39.9	702	171.5	4602
		Granular	11.8	37.5	708	158.1	5388
Flucarbazone-sodium	20	Peat	11.0	36.5	693	225.3	3578
		Granular	11.8	40.8	712	190.1	5830
Flucarbazone-sodium	30	Peat	9.0	29.7	360	144.3	2548
		Granular	11.2	37.4	666	154.1	4582
Sulfosulfuron	20	Peat	13.0	4.4	503	142.2	3952
		Granular	9.9	34.5	530	216.0	2226
Untreated check		Peat	12.0	40.3	601	160.8	3678
		Granular	9.7	33.8	651	171.1	4038
					Herbicide means		
Clopyralid + MCPA and clodinafop propargyl	660, 56		11.7	38.7	705	164.8	4988
Flucarbazone-sodium	20		11.4	38.6	7021	208.0	4704
Flucarbazone-sodium	30		10.1	33.6	512	149.2	3564
Sulfosulfuron	20		11.5	39.5	517	179.2	3058
Untreated check			10.9	37.0	626	165.9	3858
$LSD_{0.05}$			NS§	NS	NS	NS	NS
					Inoculant means		
		Peat	11.3	38.2	572	168.8	3660
		Granular	10.9	36.8	653	178.0	4412
$LSD_{0.05}$			NS	NS	NS	NS	NS

† data based on three plants per replicate.
‡ NA = nitrogenase activity.
§ NS = not significantly different according to least significant difference ($P \leq 0.05$).

Table 3.19 The impact of residual herbicides and inoculant type on final harvest seed yield and N_2 fixation parameters of field pea at Goodale (2006).

Treatment	Rate	Inoculant	Seed Yield	Seed Protein	Ndfa	Seed Ndfa	Harvest index
	g a.i. ha^{-1}		kg ha^{-1}	%	%	kg ha^{-1}	
				Herbicide × inoculant means			
Clopyralid + MCPA and clodinafop propargyl	660, 56	Peat	4280	24.7	97.4	165	0.56
		Granular	4590	24.8	96.2	174	0.56
Flucarbazone-sodium	20	Peat	4340	26.2	99.2	180	0.54
		Granular	3630	25.6	99.8	146	0.51
Flucarbazone-sodium	30	Peat	4370	25.7	99.3	178	0.55
		Granular	3780	26.1	95.5	151	0.49
Sulfosulfuron	20	Peat	4750	24.5	91.0	169	0.55
		Granular	4430	24.7	92.3	159	0.55
Untreated check		Peat	4650	24.6	99.9	182	0.56
		Granular	4930	25.1	95.6	189	0.55
				Herbicide means			
Clopyralid + MCPA and clodinafop propargyl	660, 56		4430	24.	96.8	170	0.56
Flucarbazone-sodium	20		3990	25.9	99.5	164	0.53
Flucarbazone-sodium	30		4080	25.9	97.4	165	0.52
Sulfosulfuron	20		4590	24.6	91.6	164	0.55
Untreated check			4590	24.8	97.7	186	0.56
$LSD_{0.05}$			NS	NS	NS	NS	NS
				Inoculant means			
		Peat	4480	25.3	97	175	0.55
		Granular	4270	25.1	96	164	0.53
$LSD_{0.05}$			NS†	NS	NS	NS	NS

† NS = not significantly different $P \leq 0.05$.

3.4 Discussion

3.4.1 Field assessment of the impact of pre- and post-emergent herbicide application on field pea (Experiment 1)

Experiment 1 examined the impact of pre-emergent (amitole and glyphosate) and post-emergent (MCPB + MCPA, MCPA, imazethapyr + imazamox, metribuzin and betazon) herbicides on growth and N_2 fixation in field pea. At the Clavet site, any reductions in shoot and nodule biomass as well as N accumulation 7 DAA, did not exist 29 DAA (Table 3.9). This suggests that there was herbicide injury earlier in the growing season from both pre- and post-emergent applications, but plants were able to recover from early injury. The reduction in shoot biomass likely caused the decrease in nodule biomass as the plant presumably provided less photosynthate to the roots and instead directed it to the shoots in order to recover from the damage incurred from herbicide application (Bethlenfalvay and Phillips, 1977; Gonzalez et al., 1996; King et al., 2001). These results are consistent with those of Bethlenfalvay and Phillips (1977) who carried out a field pea growth chamber experiment with the herbicide bentazon. Plants received foliar and root treatments of 1.8 kg a.i. ha^{-1} and were assayed at 6 h intervals for N_2-fixing capacity, H_2 evolution and CO_2 exchange rates. They found that the inhibition of N_2-fixing ability of the legume-*Rhizobium* relationship was not caused directly by bentazon, but indirectly by limiting the availability of photosynthate to support root-nodule activity.

In a greenhouse study, King et al. (2001) applied glyphosate as a post-emergent treatment to glyphosate-tolerant soybean. Nineteen days after application, shoot and nodule biomass and N accumulation were reduced compared to the control. Forty days after application, there were no detectable differences between any treatments. They concluded that initial nodule development in soybean was likely reduced due to a reduction in the photosynthate supply to the roots that had been re-directed to the shoots.

Nodule numbers were suppressed at the Clavet site 7 DAA, and this suppression likely resulted in decreased NA. Mallik and Tesfai (1985) reported that suppression of nodule numbers was likely caused by root hair deformations together with direct herbicidal effects on rhizobia by chemicals that inhibited photosynthesis and the

synthesis of acetolactate synthase. Both processes are important for N_2 fixation (Mårtensson and Nilsson, 1989; Mårtensson, 1992). Mårtensson (1992) reported bentazon, chlorosulfuron and glyphosate reduced nodule growth in red clover (*Trifolium pretense* L.). In the same experiment, bacterial induced root hair deformations (necessary for nodulation) decreased with increasing concentrations of bentazon, chlorosulfuron and MCPA.

Whereas herbicide treatments MPCB + MCPA reduced NA (7 DAA), efficacy of the rhizobia to fix N_2 (i.e., specific NA) remained unaffected (Table 3.9). Interestingly, when assessed 7 DAA, the NA of nodules from plants treated with amitrole and bentazon was unaffected; however, efficacy of the rhizobia increased. By the July sampling date (35 DAA), NA and specific NA were unaffected as compared to the control. Similarly, at Goodale, 7 DAA, NA was unaffected; however, herbicide treatments MCPB + MCPA and MCPA decreased specific NA. In the second sampling period (29 DAA), no differences were detected. Early measurements in which NA was unaffected, but specific NA was affected, may be attributed to a negative herbicide affect on the relationship between rhizobia and plants and thus the decrease in efficacy. Interestingly, shoot biomass was lower in these treatments (not statistically significant at $P \leq 0.05$). Perhaps photosynthate was redirected from the roots to the shoots and therefore less C was supplied to rhizobia thereby decreasing their efficacy. Also, there was a heavy weed infestation at Clavet, despite the herbicide treatments, and plots were hand-weeded. It is possible that the weed infestation caused stress on the plants and caused decreases in shoot biomass and nodule biomass at the June sampling date. By July, although the weeds continued to infest the plot, there were no differences detected in shoot biomass, nodule biomass, NA and specific NA. Any herbicide stress that the plants previously suffered was overcome as the plants recovered.

Although there was some physiological stress earlier in the season, there were no differences in yield, protein, %Ndfa, seed Ndfa, and harvest index. Pea grown at Clavet, however, achieved only very low levels of %Ndfa (Table 3.11). Pea grown at Goodale fixed N_2, but there was very high variability in %Ndfa between treatments (Table 3.12). According to EnviroTest Labs, soil results from Clavet indicated that soil N levels were "insufficient" (27 $\mu g\ g^{-1}$) (Table 3.1); therefore, it is unlikely that soil

inorganic N levels inhibited fixation as has been reported by others (Evans et al., 1989; Herridge et al. 1995; Hartwig, 1998; Clayton et al., 2004). It is possible that the heavy weed infestation at Clavet had a negative impact on crop growth such that high levels of N_2 fixation could not be supported. These findings are in agreement with other researchers who reported that weed infestations can reduce the amount of N available via N_2 fixation, although %Ndfa may increase with increasing competition for mineral N (Corre-Hellou and Crozat, 2005).

3.4.2 Field assessment of the impact of pre- and post-emergent herbicides on chickpea (Experiment 2)

Pre- and post-emergent herbicides (sulfentrazone, isoxaflutole, imazethapyr + glyphosate and metribuzin) were applied to chickpea to determine any effects on N_2 fixation. Experiment 2 included the only treatment that is registered for chickpea in Canada – metribuzin (Saskatchewan Agriculture, and Food, 2006). The 2× rate was used as a worst case scenario. Sulfentrazone is currently registered for chickpea in the US, but was only registered for chickpea in Canada in May 2008 by DuPont Canada® in an agreement with FMC Corporation (Pest Management Regulatory Agency, 2008). At the time of the field experiment, sulfentrazone was not registered. Isoxaflutole is for sale in eastern Canada and British Columbia and is registered for use in field corn. Processing pea and soybean may be cropped the following year, but no restrictions or allowances are mentioned regarding chickpea (Bayer, 2006). Imazethapyr is registered for field pea, but not chickpea (Saskatchewan Agriculture and Food, 2006).

In the first sampling period, chickpea shoot biomass and nodule mass were reduced by post-emergent metribuzin treatments. Application of pre-emergent herbicides (sulfentrazone, isoxaflutole, imazethapyr + glyphosate and metribuzin) earlier in the season did not affect chickpea shoot biomass and nodule biomass (Table 3.14). By the second sampling period 29 DAA, injured shoots recovered, but the impact of the metribuzin treatments on nodule mass persisted. Both metribuzin treatments significantly reduced nodule biomass.

Nitrogenase activity was not affected in post-emergent metribuzin treatments, when assessed at the first sampling period, even though the shoot and nodule biomass were reduced. Visual assessments indicated that early chickpea growth was slow and it is

possible that plants had not reached a state in which there was sufficient photosynthate supply to the roots to ensure effective nodulation (Rennie and Dubetz, 1984). This may explain why at the first sampling time, there were no differences in NA. Twenty-nine days after application, plants were visually damaged and there was less nodulation in the metribuzin treatments and this corresponded with a decrease in NA. However, when examining the efficacy of the nodules (specific NA) there were no differences between the treatments and the control at either sampling period. Rennie and Dubetz (1984) examined the effects of pre-emergent application of metribuzin on N_2 fixation in field pea. Although metribuzin caused erratic emergence of field pea, NA levels were higher in metribuzin treated plants than in control plants suggesting that plant growth, not nodulation or NA, suffered from metribuzin application. Research by Sprout et al. (1992) similarly suggested that any detrimental effects of metribuzin were primarily plant mediated, although the *R. leguminosarum* strain used affected the degree of inhibition. In this experiment, while there were visual differences in plant growth and differences in shoot and nodule biomass, particularly where metribuzin was applied, any decreases in NA were likely caused by plant damage. This is confirmed as the ability of the rhizobia to fix N_2 was unaffected.

Crop yields, while high, were not significantly affected by herbicide treatments according to the ANOVA (Table A.8; Table A.9; Table A 10). The 2005 autumn season had above normal temperatures and chickpea continued to flower and grow into September (Table 3.7). The high yields may be attributed to the excellent weather which encouraged shoot biomass growth and increased seed production. Chickpea was harvested after a killing frost which left the seeds shriveled and caused the pods to open up releasing seeds onto the soil. The high variability in yield (CV, 33%, data unreported) is likely because the crop failed to reach maturity, and because seed was shrunken and was lost to shattering before harvest.

3.4.3 Field assessment of the impact of residual herbicides on field pea (Experiment 3)

The impact of residual herbicide activity on nodulation, N_2 fixation and yield of field pea was examined in a two-year study initiated in 2004. During the first phase of the study, wheat was treated with herbicides (clopyralid, flucarbazone-sodium and sulfosulfuron) known to have residual properties that may carry-over the following year.

In the second phase, field pea was seeded into the wheat stubble. Field pea was inoculated with either granular (soil applied) or peat-based (seed applied) inoculant, and the potential for these inoculants to reduce the negative impact of herbicides was compared.

Saskatchewan Agriculture and Food offers crop guidelines as to when crops can/should be planted after herbicide application. Field pea may be grown the year following flucarbazone-sodium application in fields where the precipitation during the growing season has been equal to or above the 10-yr average (and where organic matter content is above 4% and pH is below 7.5) (Saskatchewan Agriculture and Food, 2006). Field pea should not be grown for at least 10 mo after clopyralid application (Saskatchewan Agriculture and Food, 2006) and there are no recropping restrictions for clodinafop-propargyl. These latter herbicides are commonly mixed (Saskatchewan Agriculture and Food, 2006). Field pea may be grown under normal moisture conditions the year following sulfosulfuron applications (Saskatchewan Agriculture and Food, 2006). The environmental conditions in the summers of 2004 and 2005 were likely favorable for microbial activity. Specifically, there were near average temperatures, but higher than normal precipitation (Table 3.7). The conditions that Saskatchewan Agriculture and Food give for field pea re-cropping on previously treated soil were met for all the field seasons and therefore it is possible there was little or no herbicide residue present.

Data supports this as the only difference detected among treatments was in 2005 (Table 3.17; Table 3.18; Table A.12; Table A.13). The only post-harvest measurements negatively affected were % Ndfa in 2005 (Table 3.17). These results were not replicated in 2006.

When examining inoculant type, Rennie and Dubetz (1984) reported that the herbicides trifluralin, metribuzin, chloramben, linuron and diclofop, did not affect nodulation or N_2 fixation of irrigated soybean grown in southern Alberta when granular inoculant was used. They state that granular inoculant applied to soil avoids intimate contact with seed-applied fungicides and limits exposure to herbicides, and therefore the inoculant is less affected. Clayton et al. (2004) determined granular inoculant increased nodule number, N accumulation and N_2 fixation compared to peat inoculant, in a field

pea experiment. Kyei-Boahen et al. (2002) examined this relationship using chickpea in a field experiment. They observed no differences in %Ndfa between peat and granular inoculants. In the 2005 field experiment, peat inoculated treatments increased NA and specific NA (Table 3.16). Denton and Pearce (2007) examined the efficacy of a variety of inoculants: peat granular, bentonite clay granular, freeze-dried inoculants used in a peat slurry on chickpea, faba bean, lentil, lupin and field pea. Peat granular and slurry inoculants resulted in equal nodulation; however, nodulation in bentonite clay granular inoculated treatments was lower than in non-inoculated treatments. Research is contradictory, and while results varied in both years of the study, it appears that peat inoculant may have some early season mitigating effects when examining N_2 fixation in conjunction with residual herbicides but only in 2005.

3.5 General Conclusions

These field experiments were designed to determine if herbicides used in western Canada applied as either a pre- or post-emergent treatment, or as soil applied herbicides with residual properties, affect N_2 fixation and consequent yield. Previous research examining the effects of herbicides on N_2 fixation has shown some herbicides may directly affect rhizobial growth (Moorman et al., 1992; Anderson et al., 2004), rhizobial survival (Singh and Wright, 1999; 2002), rhizobial recognition of the host plant (Fox et al., 2001, 2004), nodule formation (Mårtensson, 1992; Singh and Wright, 1999; Musarrat and Haseeb, 2000; Singh and Wright, 2002) and NA (Mårtensson, 1992; Anderson et al., 2004). Other research suggests that there are no direct effects on the rhizobia, but rather on plant growth (Rennie and Dubetz, 1984; Sprout et al., 1992, Gonzalez et al., 1996; Gupta, 2005). Results in this study agree with the latter research.

In Experiment 1, for early season measurements, shoot biomass was reduced by glyphosate, MCPB + MCPA, MCPA and bentazon at the Clavet site (7DAA); however, by 39DAA all differences in biomass had disappeared. At the Clavet site, nodule biomass was significantly reduced by amitrole, MCPB + MCPA, MCPA and metribuzin treatments 7DAA, but again, by 29DAA, all differences had disappeared. At both sites any reductions, in NA or specific NA in early season measurements were no longer detected in later season measurements. No differences were detected in harvest measurements including yield, protein, %Ndfa, seed Ndfa and harvest index.

In Experiment 2, chickpea was severely damaged by the herbicide treatments but only the metribuzin treatments (1×and 2× the recommended field rate) resulted in statistically significant reductions in shoot biomass (7 DAA). Nodule biomass was reduced 7DAA in the metribuzin 2× the recommended field rate and 29 DAA, both metribuzin treatments reduced nodule biomass. It was concluded that shoot biomass was under stress and likely reallocated photosynthate from the roots to the shoots. Post-harvest measurements (yield, protein, %Ndfa, seed Ndfa and harvest index) were unaffected.

In Experiment 3, residual herbicides did not reduce early season shoot biomass, N accumulation, NA, nodule biomass and specific NA. The only treatment effect detected in post-harvest measurements was %Ndfa, and only in 2005. Application of the peat-based powder inoculant limited the negative effect of the herbicide treatments on early season N_2 fixation parameters including NA and specific NA (compared to granular inoculant), but only in 2005. There were no consistent responses to herbicides or inoculant formulation between the two years.

Overall, herbicide application did not affect the ability of the rhizobia to fix N_2; however, data indicate that early season plant biomass was negatively affected by the application of some herbicides. Reduced biomass production likely reduced the photosynthate supply to the plant roots, thereby contributing to a reduction in nodule biomass. Reduced nodule establishment apparently reduced NA; however, in all the studies, the effectiveness of the rhizobia to fix N_2 (i.e., specific nitrogenase activity) was unaffected. Ultimately, final seed yield was unaffected by herbicide application in any of the experiments.

4 EFFECT OF HERBICIDES ON FIELD PEA NODULATION AND GROWTH UNDER GROWTH CHAMBER CONDITIONS

4.1 Introduction

Herbicides are applied to reduce weed competition thereby enhancing nutrient, water and light availability for the planted crops. These agrichemicals have become a vital part of modern agriculture, especially in reduced tillage systems. Herbicides control target plants by attacking specific sites and cell metabolism within the plants. Typically enzyme and protein pathways in the plant are disrupted (Hall et al., 1999). The herbicide interferes with specific enzymes or proteins thereby disrupting normal plant functions such as photosynthesis or amino acid synthesis. Unfortunately, enzyme pathways in plants such as the acetolactate synthase (ALS) pathway or the acetohydroxy acid synthase (AHAS) pathway also are present in microorganisms such as rhizobia (Royuela et al., 1998). Because of these shared pathways, and the symbiotic relationship between rhizobia and legumes, it is plausible that some herbicides might negatively affect this symbiotic relationship.

Different herbicides may affect the legume-*Rhizobium* association differently. For example, the herbicide flucarbazone-sodium is an ALS/AHAS inhibitor that binds to these enzymes and disrupts amino acid synthesis. Clopyralid and MCPA and clodinafop-propargyl similarly have the potential to negatively affect N_2 fixation. Clopyralid + MCPA are auxins that mimic indole acetic acid (IAA). These herbicides regulate plant development including apical dominance, cell division and leaf expansion (Hall et al., 1999). Clodinafop-propargyl inhibits the enzyme acetyl CoA carboxylase (ACCase) thereby inhibiting fatty acid biosynthesis (Hall et al., 1999).

In cropping systems, target plants are controlled by the use of herbicides; however non-target organisms also may be negatively affected. Anderson et al. (2004) claim that herbicides may negatively affect the legume-*Rhizobium* relationship by: (1) directly affecting root and shoot biomass of the host plant thereby limiting the number of

available sites for rhizobia to attach to or by decreasing the carbohydrate supply to existing nodules; (2) directly affecting rhizobial survival or growth that leads to a decreased potential for rhizobial infection on root hairs; (3) inhibiting or inactivating the biochemical signaling that plants require to initiate nodule development – this inhibition could affect either rhizobia or plants; and (4) inhibiting nodule development by reducing the capacity for cell division.

A number of studies have shown that herbicides may inhibit nodulation (Mallik and Tesfai, 1985; Eberbach and Douglas, 1989; Mårtensson and Nilsson, 1989; Isoi and Yoshida, 1990) and N_2 fixation (De Felipe et al., 1987; Eberbach and Douglas, 1991; Mårtensson, 1992; Koopman et al., 1995; Musarrat and Haseeb, 2000). For example, Mårtensson (1992) studied bentazon (photosynthetic inhibitor), chlorosulfuron (ALS/AHAS inhibitor) and MCPA (IAA mimic). Using Jensen's N-free media to grow red clover (*Trifolium pretense* L.cv. Britta), lucerne (*Medicago sativa* L.cv. Vertus) and birdsfoot-trefoil (*Lotus corniculatus* L.), these herbicides triggered growth disorders such as root hair deformations that inhibited symbiosis and resulted in fewer nodules. The reason for the inhibitory effect was that these chemicals inhibit photosynthesis and acetolactate synthesis, both important for N_2 fixation. However the low number of nodules associated with chlorosulfuron application was attributed to an impedance of nodule formation and not nodule initiation (low nodule weights point to an effect on nodule development or maintenance).

Some herbicides used in cropping systems such as amitrole, flucarbazone-sodium, and clopyralid, are known to have residual or carry-over properties (Eberbach and Douglas, 1991; Eliason et al., 2004; Johnson, 2004; Enloe et al., 2005). If herbicides remain in the soil until the following cropping year, there may be an opportunity for a negative interaction between the herbicide residue and N_2 fixation. Eberbach and Douglas (1991) applied amitrole and 2,4-D at rates of 0, 2, 5 and 10 µg a.i. g^{-1} soil to a sandy loam soil and allowed it to degrade for 120 d. Amitrole at rates higher than 2 µg a.i. g^{-1} soil carried over and were lethal to sub-clover (*Trifolium subterraneum* L.) seedlings. Plant growth, nodulation and NA of plants also were reduced. Application of 2,4-D similarly reduced plant growth and nodule formation, but NA was unaffected.

Under field conditions, the amount of residual herbicide persisting in the soil can be

highly variable. Depending on environmental conditions, microbial decomposition of herbicides may be favored in a given year and little or no herbicide residue may be detected in the soil (Johnson, 2004). In our field experiments (Chapter 3), we were unable to predict the levels of herbicide carry-over with any certainty. Therefore, a growth chamber experiment was initiated to investigate the impact of known amounts of simulated herbicide residues on initial stages of N_2 fixation in field pea. In particular, soil was spiked with known levels of clopyralid + MCPA and clodinafop-propargyl, and flucarbazone-sodium.

Clopyralid + MCPA and clodinafop-propargyl (trade name: Curtail M® provided by Dow AgroSciences and Horizon® provided by Syngenta) is a common tank mix used on the prairies that uses synthetic auxins and ACCase inhibitors to kill target weeds. It is recommended that field pea not be grown for at least ten months after application and not if the application year was dry (Saskatchewan Agriculture and Food, 2006). Flucarbazone-sodium (trade name: Everest® provided by Arysta) inhibits ALS/AHAS and may carry-over to the following year (Eliason et al., 2004). According to current recommendations, field pea may be grown the year following application only if precipitation has been equal to, or above, the ten-yr average during the growing season and where soil organic matter is greater than 4% and pH is below 7.5 (Saskatchewan Agriculture and Food, 2006). This experiment was conducted to determine the levels at which clopyralid + MCPA and clodinafop-propargyl as well as flucarbazone-sodium, negatively affect N_2 fixation. Additionally, the impact of peat-based inoculant on the symbiotic association was investigated and compared to uninoculated plants to determine possible mitigating effects.

4.2 Materials and Methods

Two experiments were conducted simultaneously. The first experiment studied the effect of the herbicides clopyralid + MCPA and clodinafop-propargyl on N_2 fixation by inoculated and uninoculated pea. The second experiment examined the impact of flucarbazone-sodium.

An Orthic Dark Brown Chernozem soil (Bradwell association) was collected from the 0- to15-cm depth at Goodale in September, 2005. The field was previously planted to wheat in 2005 and treated with clodinafop-propargyl and bromoxynil + MCPA

(Buctril M® provided by Bayer CropScience), both of which are considered to be non-residual herbicides. The soil was initially dried and passed through a 4 mm sieve to remove large pieces of organic material. Samples were bulked and subsamples were sent to EnviroTest Labs (ALS Laboratory Group, Saskatoon, SK) for standard soil analysis.

Soil analyses included pH and E.C. [determined on a 1:2 soil water suspension (Hogg and Henry, 1984)], inorganic N (NO_3^-) [measured by KCl extraction (Maynard and Kalra, 1993)], phosphorus, as P_2O_5, and potassium as K_2O [measured using the modified Kelowna extraction method (Qian et al., 1994)]. Selected soil characteristics such as fertility (N, P and K) as well as E.C. and pH are described in Table 4.1.

Table 4.1 Characteristics of the soil used in the growth chamber experiment.

Depth	Texture	pH	E.C.	N	P	K
cm			mS cm^{-1}		μg g^{-1}	
0-15	Loam	7.3	0.2 (non-saline)	8	>67	>672

The growth chamber experiments were conducted using a 16-h light cycle at 21°C (light intensity of approximately 45 600 lx), and an 8-h dark cycle at 15°C, with ambient relative humidity.

Five herbicide rates were used to mimic a range of soil residual levels: 0.0, 0.0625, 0.125, 0.25, 0.5 and 1× the recommended field application rates. Conversion of field application rates to mg pesticide g^{-1} air-dry soil was calculated assuming an even distribution of the pesticide in the plough layer to 10 cm. This is by necessity a simplification of the natural situation where a concentration gradient determined by the nature of the herbicide and the soil type may exist. Treatments were replicated four times.

For this study, 15-cm pots were lined with plastic bags to prevent any leaching of nutrients. Each pot contained the air dry equivalent of 1200 g of soil. Twenty milliliters of micronutrient solution were pipetted onto the soil surface to deliver 0.6 μg g^{-1} Mo as

$NaMO_4 \cdot 2H_2O$, 1.5 μg g^{-1} B as H_3BO_3, 5 μg g^{-1} Mn as $MnSO_4 \cdot H_2O$, 4 μg g^{-1} Zn as $ZnSO_4 \cdot 7H_2O$ and 0.6 μg g^{-1} Cu as $CuSO_4 \cdot 5H_2O$. Twenty milliliters of K, P and S solution were added to deliver 200 μg g^{-1} K as KCl and K_2SO_4, 30 μg g^{-1} of P as $Ca(H_2PO_4) \cdot H_2O$ and 50 μg g^{-1} of S as K_2SO_4. This ensured that other nutrients would not be limiting. Nutrients were thoroughly mixed into the soil. The following day herbicides were added to the soil by pipetting an aqueous solution onto the soil surface, and mixing thoroughly.

In the clopyralid + MCPA and clodinafop-propargyl experiment, the recommended rate is 99 g a.e. ha^{-1} clopyralid and 554.4 g a.i. ha^{-1} MCPA which is premixed as Curtail M®. Treatment rates were equivalent to 0.0, 0.114, 0.228, 0.456, 0.912 and 1.82 μL per pot. For clodinafop-propargyl, applied field rates were 0.0, 0.013, 0.027, 0.054, 0.107 and 0.214 μL per pot (1× rate is 55.7 g a.i. ha^{-1}). In the flucarbazone-sodium experiment, rates added to the soil included 28 μg a.i. per pot (in 100 mL solution) for the 1× rate while subsequent rates were 14, 7, 3.5, 1.75 and 0.0 μg a.i. per pot (in 100 mL solution). Following herbicide addition and thorough mixing, H_2O was added to bring the soil to 65% field capacity. Five pea seeds (cv. CDC Mozart) were planted 6 cm deep in each pot and all but two seedlings were pulled out after germination. Half of the pots received seed treated with peat-based inoculant (N-Prove® by PhilomBios) at an inoculation rate of 1.6 g kg^{-1} seed while the remaining pots were seeded with uninoculated seed. To reduce moisture loss from evaporation, 50 g of polypropylene beads were laid on the soil surface. Pots were randomly re-arranged and weeded two times per week and, after germination, were watered daily to 75% field capacity. During peak N accumulation (49 DAP - between flowering and pod-filling stage), plants were harvested. Shoot biomass, and washed roots and nodules were dried for 72h at 45°C and weighed.

At harvest, ARAs were performed to determine the N_2-fixing activity (Hardy et al., 1973). Acetylene is reduced to ethylene by the nitrogenase enzyme in the nodules (Azam and Farooq, 2003). Two plants were removed from the pot and shaken gently. Shoots were cut off and the exposed roots were placed in a 1 L Mason jar. One-hundred millilitres of headspace were removed using a 40 mL syringe and replaced with 100 mL of acetylene (C_2H_2). Jars were intermittently shaken to maximize nodule exposure to

acetylene. Before sampling, air in the jars was mixed by pumping the syringe four to five times to ensure a homogeneous mixture of the gases. Following incubation for 30 min, 20 mL of headspace gas were removed and placed into 12 mL vacutainers (Labco Ltd., Buckinghamshire, U.K.). Samples were analyzed using a gas chromatograph (Hewlett-Packard 5890A) fitted with a flame ionization detector (FID) for the concentration of nitrogenase-reduced acetylene. Chromotagraphic separation was carried out on a 1.8m Porplak™Q column (80/100 mesh), and the instrument was run at 60°C with N as the carrier gas at a flow rate of 40 cc min^{-1}.

Total N content in roots and shoots was analyzed using a LECO CNS-2000 analyzer. Protein content was calculated using percent N and a conversion factor of 6.25 (Sosulski and Imafidon, 1990).

4.3 Statistical Analysis

The data were analyzed using SPSS 14.0 for Windows to determine normality (SPSS 14.0, 2005). In cases where data were not normal, data were transformed using the log function (Bland and Altman, 1996). Because the LSD (at the 5% level of probability) rankings were unchanged by transformations, statistical analyses are reported for untransformed data. The data set was subjected to a two-way ANOVA using the General Linear Model in SAS and LSD ($P \leq 0.05$) was used to determine differences between treatments (SAS Institute, 1996).

4.4 Results

This study was designed to examine the impact of residual herbicides clopyralid + MCPA and clodinafop-propargyl and flucarbazone-sodium, on N_2 fixation in peat-based powder inoculated and uninoculated pea plants.

4.4.1 Clopyralid + MCPA and clodinafop-propargyl

Visual assessments of plants grown in soil spiked with clopyralid + MCPA and clodinafop-propargyl revealed typical herbicide damage symptoms (Hall et al., 1999) including stunting, chlorosis and epinasty (cupping and twisting of leaves), particularly for the 1× rate. Visually, plant growth suppression was greatest in plants treated with the highest herbicide rates (Fig 4.1). The ANOVA revealed that both herbicide and inoculant treatments affected shoot biomass, but there was no interaction

Figure 4.1. Inoculated pea planted in clopyralid +MCPA and clodinafop-propargyl treated soil. Treatments from left to right are: control; 1×; 0.5×; 0.25×; 0.125×; and 0.0625× the recommended application rate.

between these variables (Table 4.2).

Both the 1× and the 0.5× herbicide rates reduced shoot biomass as compared to the control (Table 4.2; Fig 4.2; Table A.15). Plants were not affected by the 0.25×, 0.125× and 0.0625× herbicide rates. Inoculation significantly increased shoot biomass (Fig 4.2; Table A. 15).

A significant effect of herbicide application was detected for all measured parameters (Fig 4.2; Fig 4.3; Fig 4.4; Table 4.2; Table A.15; Table A.16). Typically only the full rate, and in one instance application of the 0.5× rate, significantly affected the measured parameters (e.g. shoot weight). The effect of the herbicide was generally observed as a reduction in the measured parameters with the exception of increases in concentration of N in the shoots and specific NA in the full rate (Fig 4.5; Fig 4.6).

Inoculation significantly affected shoot biomass, root N concentration, NA and nodule biomass (Table 4.2; Table A.15; Table A.16). Specifically, these variables were enhanced by inoculation.

Although both herbicide treatments and inoculation influenced various growth parameters, interactions between herbicides and inoculants were detected only for root N concentration, NA, and root biomass (Table 4.2; Fig 4.3; Fig 4.4; Fig 4.7; Table A.15; Table A.16). Specifically, root N concentration and NA increased with increasing concentrations of the herbicide, but only for inoculated plants. Interactions affecting root biomass varied. At the 0.0625× rate, inoculation was associated with reduced root biomass, whereas at other herbicide rates, there were no detectable differences.

4.4.2 Flucarbazone-sodium

Plants grown in soil spiked with flucarbazone-sodium at 1× and 0.5× the recommended field rate were stunted and thin (Fig 4.8). Biomass measurements confirmed this visual assessment (Table 4.3) and plant growth improved with decreasing herbicide rates (Fig 4.9; Table A.17). Both the 1× rate and 0.5× herbicide rate resulted in decreased shoot weight (Table 4.3; Fig 4.9; Table A.17; Table A. 18). Inoculation did not significantly affect shoot biomass, nor was an interaction between the herbicide and inoculation treatments detected.

Table 4.2 ANOVA information for the growth chamber experiment examining the impact of different rates of clopyralid + MCPA and clodinafop-propargyl on N_2 fixation.

	Shoot biomass				Shoot N concentration				Root biomass				Root N concentration			
	g				mg				%				%			
	df	MS	F	*P*	df	MS	F	*P*	Df	MS	F	*P*	df	MS	F	*P*
Model	11	7.855	63.14	0.000	11	0.795	6.313	0.000	11	0.188	12.83	0.000	11	0.414	5.98	0.000
Herbicide	5	16.961	136.35	0.000	5	1.511	11.994	0.000	5	0.327	22.3	0.000	5	0.350	5.06	0.001
Inoculant	1	0.706	5.67	0.023	1	0.078	0.616	0.438	1	0.004	0.271	0.606	1	1.287	18.59	0.000
Herbicide * Inoculant	5	0.179	1.44	0.233	5	0.223	1.771	0.144	5	0.086	5.851	0.000	5	0.304	4.39	0.003
Error	36	0.124			36	0.126			36	0.015			36	0.069		

	NA				Nodule biomass				Specific NA			
	µmol ethylene h^{-1}				mg				µmol ethylene g^{-1} nodule h^{-1}			
Source of variation	df	MS	F	*P*	df	MS	F	*P*	Df	MS	F	*P*
Model	11	19 709	7.15	0.000	11	0.141	14.82	0.000	11	850 663	1.977	0.061
Herbicide	5	28 921	10.49	0.000	5	0.275	28.97	0.000	5	1 655 990	3.849	0.007
Inoculant	1	35 940	13.04	0.001	1	0.087	9.17	0.005	1	106 089	0.247	0.623
Herbicide * Inoculant	5	7 250	2.63	0.040	5	0.017	1.80	0.137	5	194 251	0.451	0.809
Error	36	2 757			36	0.009			36	430 290		

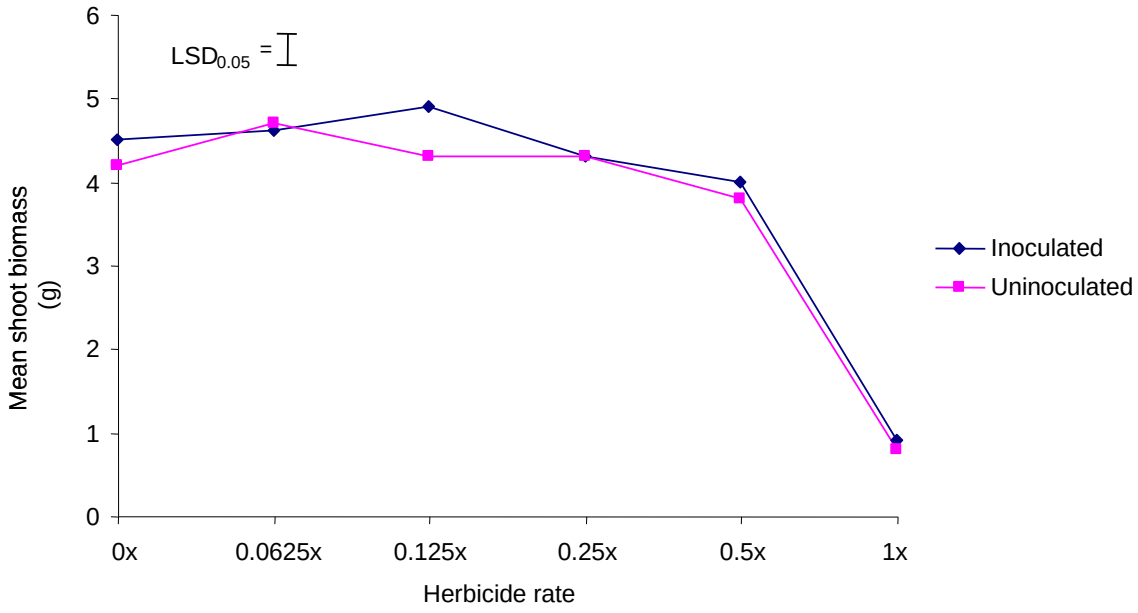

Figure 4.2 Mean shoot biomass of pea planted in soil spiked with clopyralid +MCPA and clodinafop-propargyl.

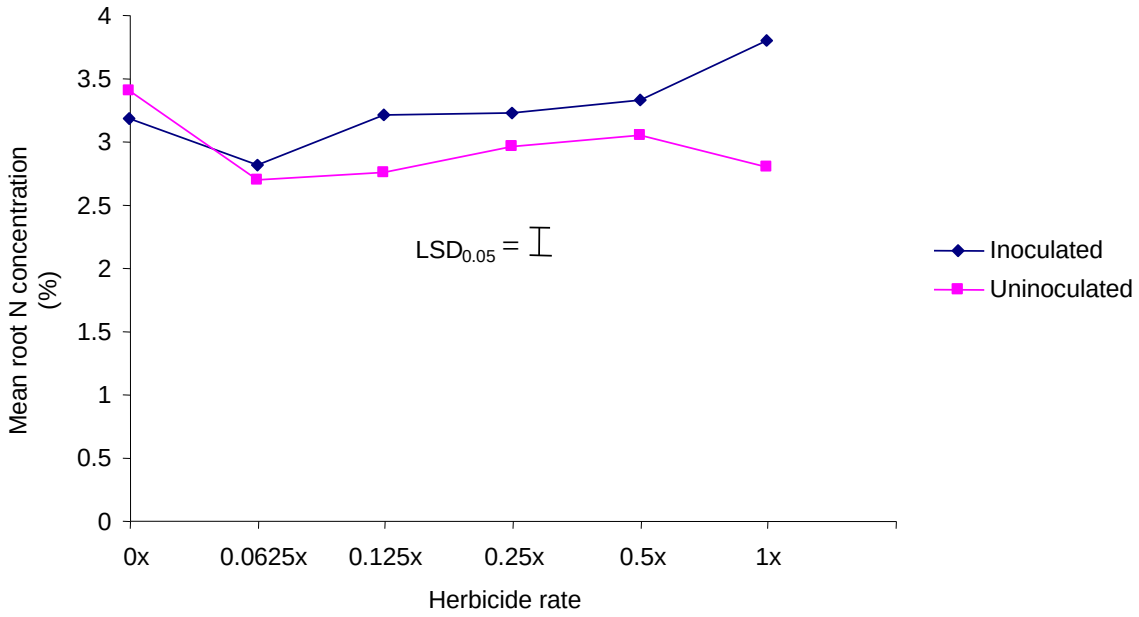

Figure 4.3 Mean root N concentration in pea planted in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

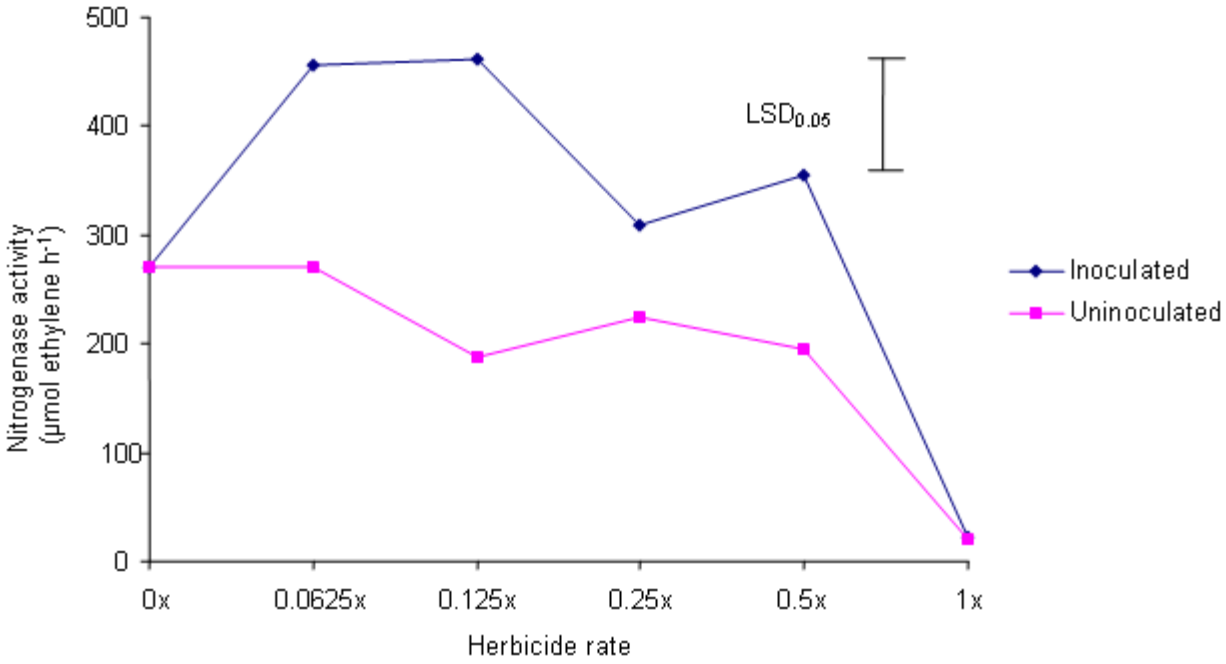

Figure 4.4 Nitrogenase activity in pea planted in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

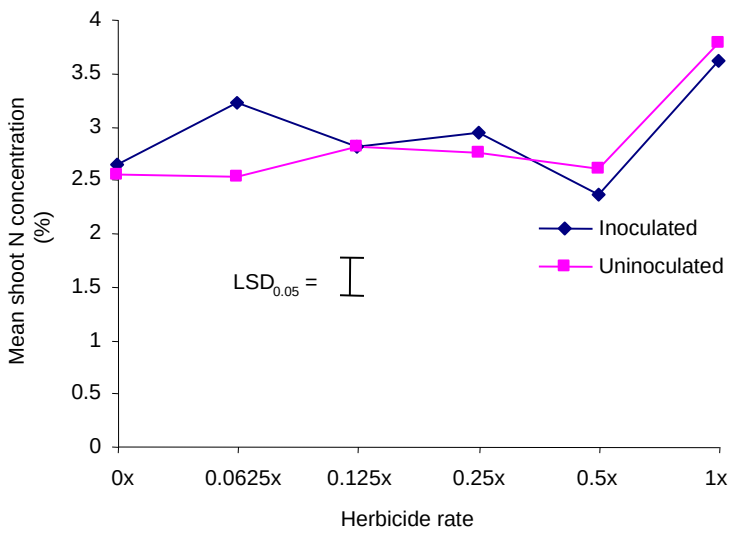

Figure 4.5 Mean shoot N concentration in pea planted in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

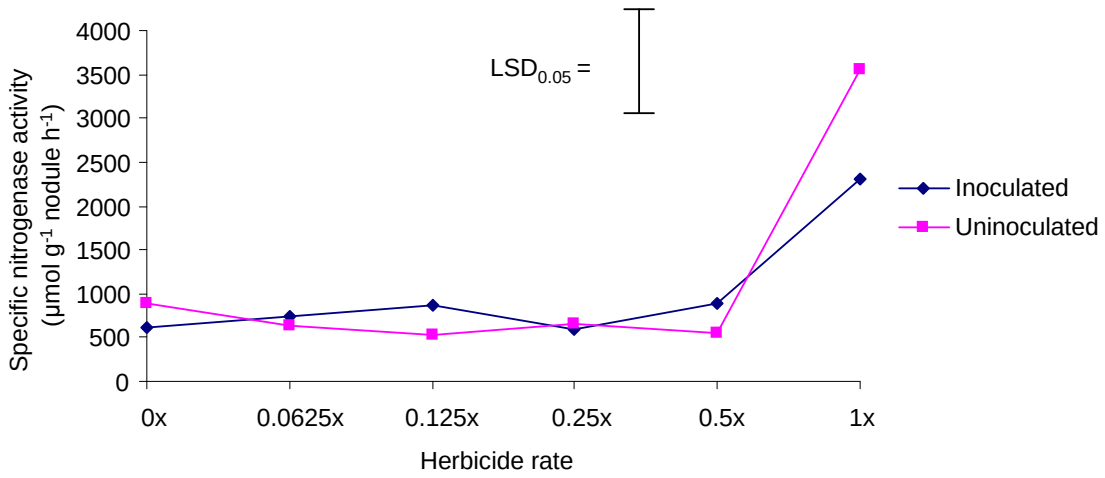

Figure 4.6 Specific nitrogenase activity in pea planted in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

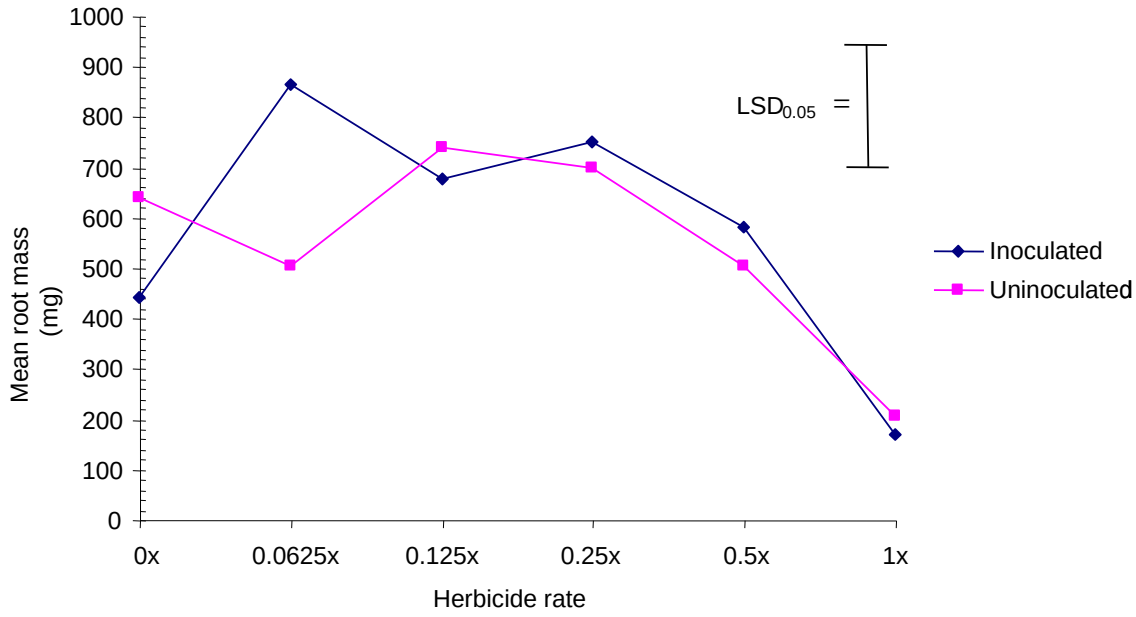

Figure 4.7 Mean root mass in pea planted in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

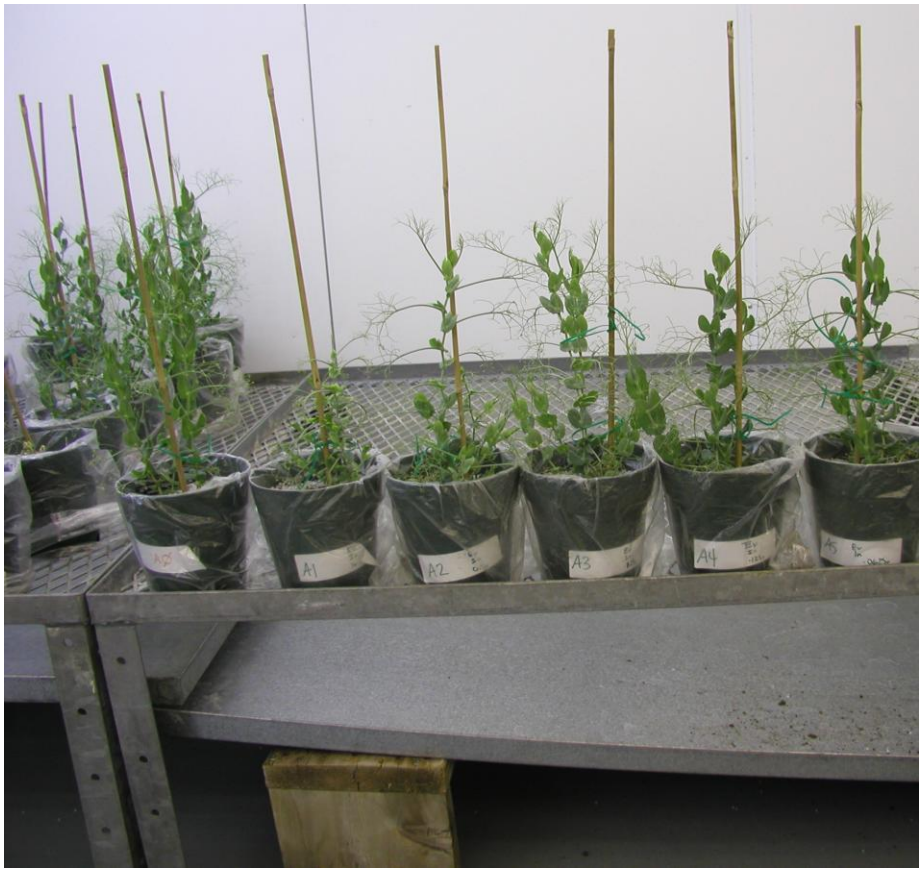

Figure 4.8 Inoculated pea planted in flucarbazone-sodium treated soil. Treatments from left to right are: control; 1×; 0.5×; 0.25×; 0.125×; and 0.0625× the recommended application rate.

Table 4.3 ANOVA information for the growth chamber experiment examining the impact on N_2 fixation by different rates of flucarbazone-sodium.

	Shoot biomass				Shoot N concentration				Root biomass				Root N concentration			
	g				mg				%				%			
	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	11	4.841	13.445	0.000	11	1.106	3.860	0.001	11	0.063	3.597	0.002	11	0.115	1.606	0.139
Herbicide	5	10.239	28.438	0.000	5	2.217	7.736	0.000	5	0.044	2.532	0.046	5	0.210	2.935	0.025
Inoculant	1	0.057	0.159	0.692	1	0.119	0.415	0.523	1	0.143	8.194	0.007	1	0.044	0.613	0.439
Herbicide * Inoculant	5	0.399	1.108	0.373	5	0.192	0.672	0.647	5	0.065	3.743	0.008	5	0.034	0.475	0.792
Error	36	0.360			36	0.287			36	0.017			36	0.071		

	NA				Nodule biomass				Specific NA			
	µmol ethylene h^{-1}				mg				µmol ethylene g^{-1} nodule h^{-1}			
Source of variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	11	6 745	1.733	0.105	11	0.059	5.108	0.000	11	202 050	8.103	0.000
Herbicide	5	10 601	2.724	0.035	5	0.126	10.952	0.000	5	399 861	16.036	0.000
Inoculant	1	6 056	1.556	0.220	1	0.004	0.314	0.579	1	72 570	2.910	0.097
Herbicide * Inoculant	5	3 026	0.778	0.572	5	0.003	0.224	0.950	5	30 134	1.208	0.325
Error	36	3 891			36	0.012			36	24 936		

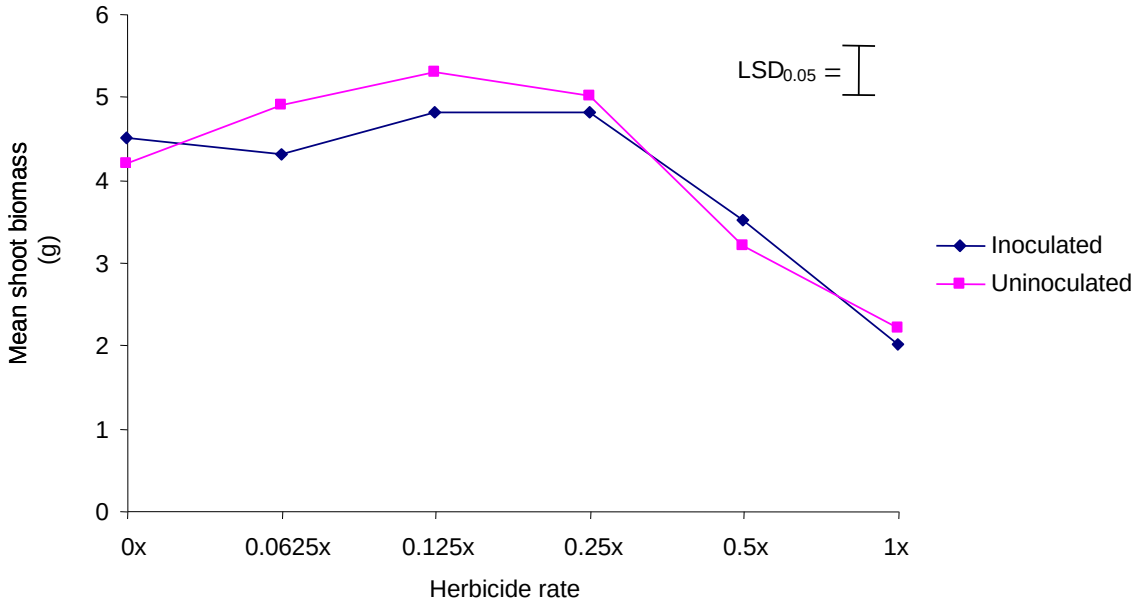

Figure 4.9 Mean shoot biomass of pea planted in soil spiked with flucarbazone-sodium.

The ANOVA revealed that all measured parameters were significantly affected by herbicide application (Table 4.3; Fig 4.9; Fig 4.10; Fig 4.11; Fig 4.12; Fig 4.13; Fig 4.14; Table A.17; Table A.18). Generally, the 1× rate and for some parameters, the 0.5× rate, caused a significant effect (Table 4.3; Table A. 17; Table A.18). The herbicide caused a reduction in measured parameters, with the exception of shoot N concentration and specific NA (Fig 4.9; Fig 4.14). Root biomass was the only parameter where differences were detected between inoculated plants and uninoculated plants. It was also the only measurement where there was an interaction detected between the treatments and the inoculant (Fig 4.11). Specifically, root biomass of inoculated plants increased in soil treated with herbicide at rates greater or equal to 0.0625×. In the control, uninoculated plants had greater root biomass.

4.1 Discussion

4.1.1 Clopyralid +MCPA and clodinafop-propargyl

Shoot and root growth was restricted at the 1× rate of clopyralid +MCPA and clodinafop-propargyl (Fig 4.2; Fig 4.7). Clopyralid + MCPA and clodinafop-propargyl did not kill the plants, but severely reduced the biomass. The reduced shoot and root biomass is reflected in a similar decrease in nodule mass and nodule number (Table 4.2; Table A.15; Table A.16). The correlation between plant growth and nodulation is well documented (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977; Rennie and Dubetz, 1984; Eberbach and Douglas, 1991; Sprout et al., 1992; Daniel et al., 1999; Gupta, 2005). Symbiotic N_2 fixation depends on *Rhizobium* receiving C energy derived from photosynthesis from the plant (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977). In this experiment, decreases in shoot and root biomass are likely linked and the reduction in shoot biomass may have caused a reduction in root biomass, or vice versa. Rhizobial infection of legumes takes place through root hairs and the process of nodulation is linked to the surface area and root length (Pate, 1977). Clopyralid + MCPA and clodinafop-propargyl reduced nodule mass possibly by restricting photosynthate availability (e.g., at the 1× rate). Root growth was also inhibited and may have decreased the number of potential infection sites for the rhizobia and as a

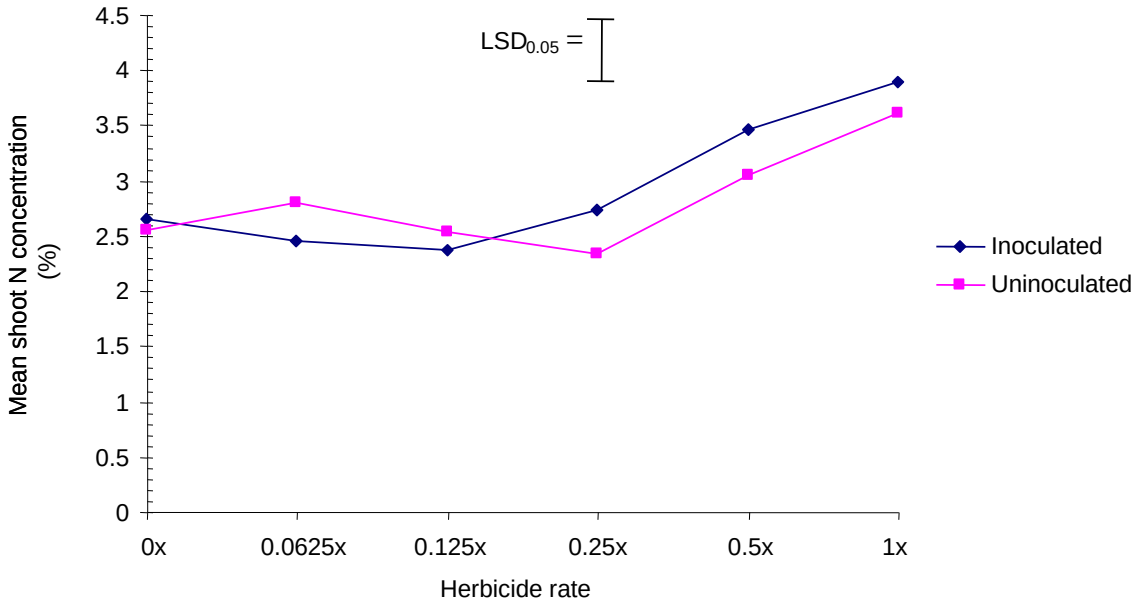

Figure 4.10 Mean shoot N concentration of pea planted in soil spiked with flucarbazone-sodium.

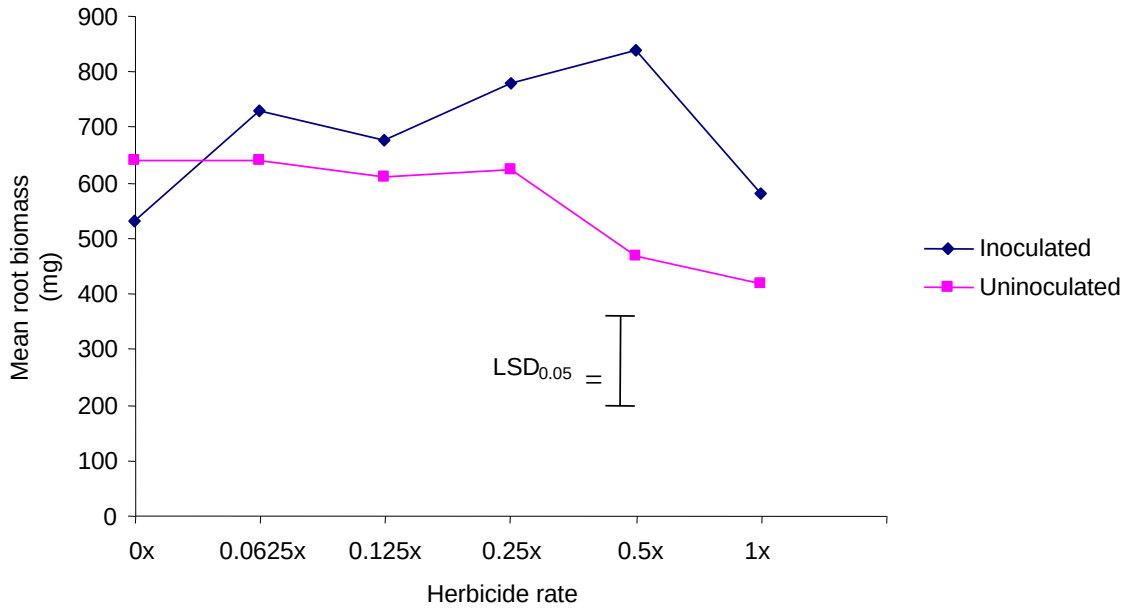

Figure 4.11 Mean root biomass for pea planted in soil spiked with flucarbazone-sodium.

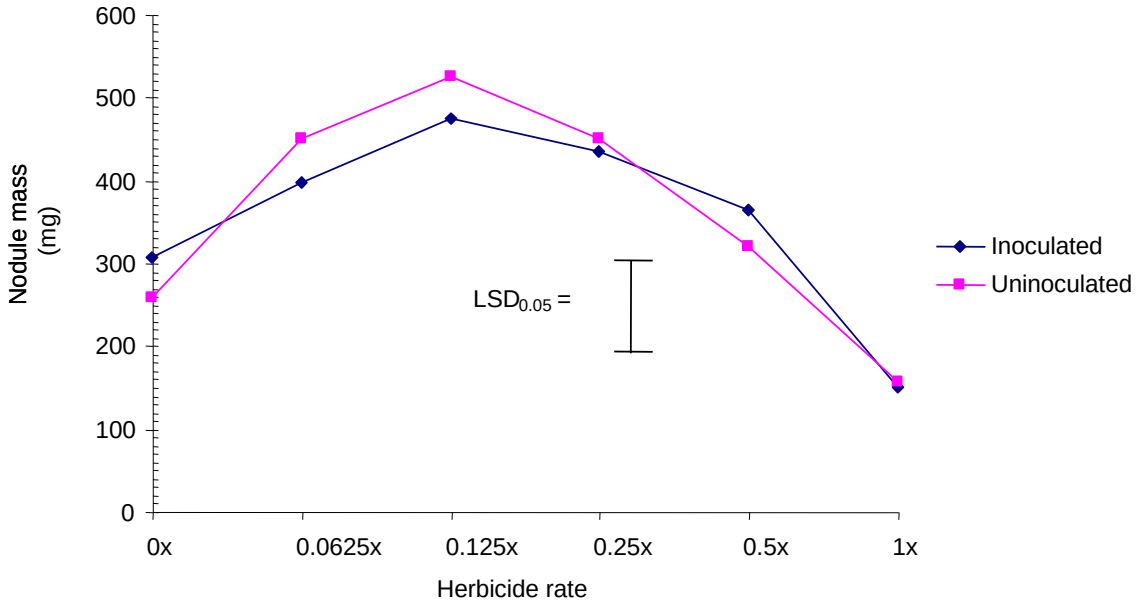

Figure 4.12 Mean nodule mass for pea planted in soil spiked with flucarbazone-sodium.

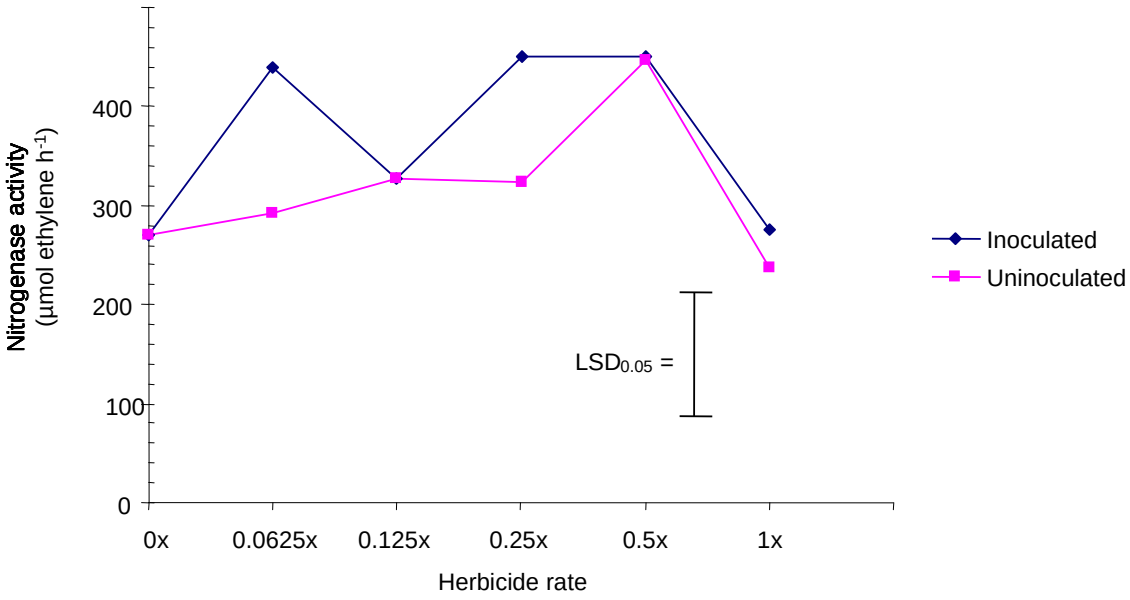

Figure 4.13 Nitrogenase activity for pea planted in soil spiked with flucarbazone-sodium.

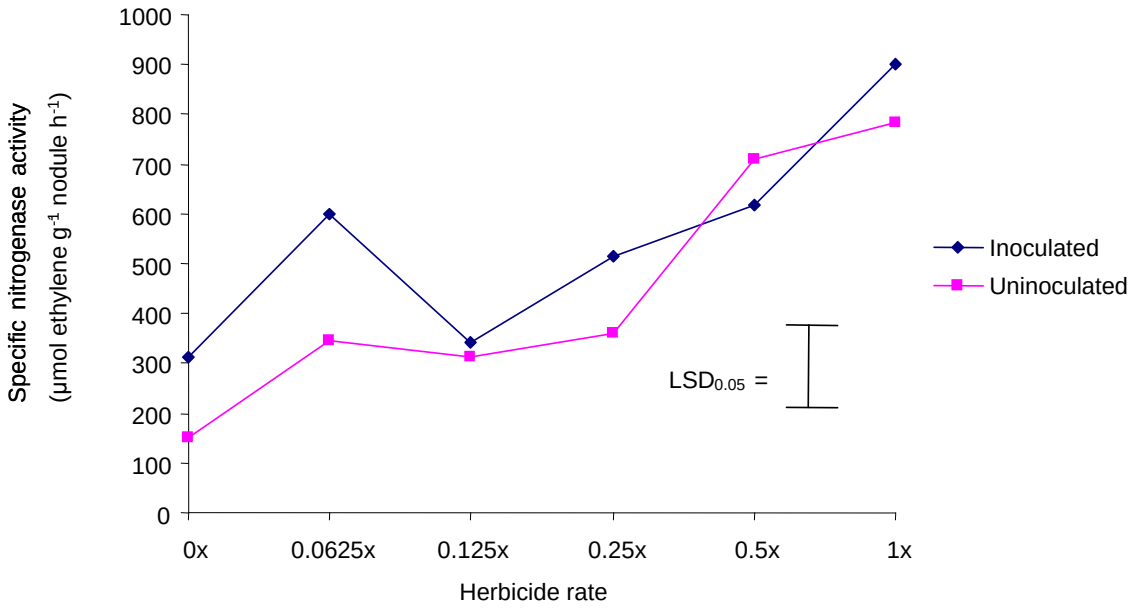

Figure 4.14 Specific nitrogenase activity for pea planted in soil spiked with flucarbazone-sodium.

consequence, nodule mass decreased in the 1× treatment. The reduction in nodule number and mass in pea planted in the 1× rate likely was not due to a specific effect of the herbicide on the rhizobia, but rather on the process of nodule establishment due to a lower root biomass (Mallik and Tesfai, 1985; Eberbach and Douglas, 1991) and a lack of available photosynthate supply (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977; Rennie and Dubetz, 1984; Eberbach and Douglas, 1991, Sprout et al., 1992; Daniel et al., 1999; Gupta, 2005).

Conversely, there was an increase in shoot N concentration in the 1× rate of clopyralid + MCPA and clodinafop-propargyl (Table A.15). Higher N concentration in the shoots suggests that N uptake was not limiting to plant growth whereas herbicide application restricted further plant development and hence N became concentrated in the plant tissue (Table A.15).

It was particularly interesting to note that nodule mass, nodule number and NA were lowest in pea exposed to the 1× rate, but specific NA was the highest at the full rate (Fig 4.4; Table A.15; Table A.16). This indicated that rhizobia were effective in fixing N_2. The increase in the ability of the rhizobia to fix N_2 may be a means by which the rhizobia attempted to compensate for poor nodulation (Singh and Wright, 1999).

The aim of inoculation is to increase the numbers of viable rhizobia in the plant rhizosphere, thereby increasing nodulation and N_2 fixation to increase yield (Thies et al., 1991). In this experiment, peat inoculation significantly enhanced shoot biomass, root N concentration, nodule mass and NA (Table A.15; Table A.16). Flores and Barbachano (1992) examined the sensitivity of rhizobia to herbicides. They found three strains of *Rhizobium meliloti* to be more sensitive in Gramoxone® than Diuron® or Totacol®. The rhizobial strain (peat inoculant) used in this experiment did not appear to be sensitive to clopyralid + MCPA and clodinafop-propargyl and was more efficient in NA than indigenous rhizobia (i.e., uninoculated plants).

Although both herbicide treatment and inoculation influenced various growth parameters, interactions between these two factors were only detected for root biomass, root N concentration and NA. Mårtensson (1992) found an interaction between the herbicide MCPA and inoculation. In his study, lower concentrations of herbicide

stimulated the number of nodules on inoculated red clover roots. Clopyralid and MCPA are synthetic auxins that regulate plant growth. At lower concentrations, the herbicide may have acted as a stimulant to the plant and the inoculated or uninoculated plants may have responded to the stimulus. Therefore, inoculation may have mitigated the negative effects of herbicide treatment on parameters such as NA.

4.1.2 Flucarbazone-sodium

Flucarbazone-sodium blocks the ALS enzyme thus halting the production of branched-chain amino acids. This effectively leads to plant starvation and death. While no plants died in the experiment, shoot biomass of plants grown in 1× rate and 0.5× rate was decreased (Table 4.3; Fig 4.9; Table A.17; Table A.18). Similarly, nodule mass and nodule number decreased in pea planted in the 1× rate. As mentioned in the discussion relating to the clopyralid + MCPA and clodinafop-propargyl experiment, the relationship between plant growth and nodulation is clear (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977; Rennie and Dubetz, 1984; Eberbach and Douglas, 1991; Sprout et al., 1992; Daniel et al., 1999; Gupta, 2005). The decrease in nodule mass and nodule number is related to the plant's ability to supply C to the roots. If the plant is stressed, C will be re-directed to the shoots. Although no differences were detected in root biomass, the roots may not have supplied a sufficient amount of C to the rhizobia and thus the decrease in nodule mass in the 1× rate (Hardy and Halvelka, 1975; Bethlenfalvay and Phillips, 1977; Rennie and Dubetz, 1984; Eberbach and Douglas, 1991; Sprout et al., 1992; Daniel et al., 1999; Gupta, 2005).

Again, similarly to the clopyralid + MCPA and clodinafop-propargyl experiment, there was an increase in shoot N concentration in pea at the 1×rate (Fig 4.10; Table A.17). The higher N concentration in the shoots indicates that N uptake did not limit plant growth, but the full application rate of flucarbazone-sodium caused a decrease in shoot biomass. This caused N to be concentrated in plant tissue.

Shoot biomass, nodule mass, and NA were lowest in pea planted in the 1× rate (Fig 4.9, Fig 4.12; Fig 4.13; Table 4.3; Table A.17; Table A.18), but specific NA was the highest. Again, this indicated that rhizobia were effective in fixing N_2 and there was no evidence that rhizobial function was negatively influenced by the herbicide application.

When comparing inoculated plants to uninoculated plants, only root biomass differed

significantly (Fig 4.11). Specific parameters such as shoot biomass, nodule number and mass, and NA were unaffected by inoculation. It is possible that the inoculant strain was sensitive to flucarbazone-sodium (Flores and Barbachano, 1992) and this is the reason that there was no increase in NA in inoculated plants. Specific NA did not differ in inoculated and inoculated plants, and therefore the strain was neither more nor less effective in fixing N_2. Inoculation had no mitigating effects on any other measured parameters (except root biomass).

4.2 General Conclusions

Herbicide application has become a routine part of farming on the prairies. Generally herbicides degrade in soil, but there are some that are known to have residual or carry-over properties into the following year. This experiment was designed to study the impact of residual herbicides on N_2 fixation in field pea. The growth chamber experiment used a range of herbicide rates that were based on the assumption that the herbicide normally penetrated to a depth of 10 cm and was homogenous to this depth. This is a very unlikely field situation and provided a worst case scenario. The two experiments examined a herbicide tank mix of clopyralid + MCPA and clodinafop-propargyl, and the herbicide flucarbazone-sodium. Results were similar in both experiments – the greatest damage was in pea planted in the 1× rate. Where plant biomass was reduced, and rhizobial functioning (NA) negatively affected, it is likely that the latter occurred due to reduced allocation of C to the nodules. Specific NA was significantly higher in the 1× rate compared to all treatments. This indicates that rhizobia that infected the plants were able to fix N_2, irrespective of the herbicide treatment. In the clopyralid + MCPA and clodinafop-propargyl experiment, inoculant enhanced shoot biomass, root N concentration, nodule mass and NA, but the only interactions between the herbicide and inoculant treatments were detected for root biomass, root N concentration and NA. Within these parameters, peat inoculation may mitigate the negative effects of herbicides on N_2 fixation when compared to uninoculated plants. In the experiment with the herbicide flucarbazone-sodium, however, no mitigating effects on N_2 fixation were detected and peat inoculation only increased root biomass

Results from this growth chamber experiment indicate that only under extreme

conditions, not normally found in the field, would herbicides such as clopyralid + MCPA and clodinafop-propargyl and flucarbazone-sodium negatively affect N_2 fixation in field pea.

5 IMPACT OF HERBICIDES ON RHIZOBIAL GROWTH UNDER LABORATORY CONDITIONS

5.1 Introduction

Herbicides can influence the success of the legume-*Rhizobium* symbiosis either by affecting the plant, the rhizobia or both (Anderson et al., 2004). Although many researchers have concluded that the impact of herbicide application on the symbiotic partnership is due largely, or exclusively, to direct effects of the herbicide on plant growth and consequent photosynthate allocation to the nodules (Rennie and Dubetz, 1984; Sprout et al., 1992; Vidal et al., 1992, Abd-Alla et al., 2000; Erman et al., 2004), others have argued that herbicides may inhibit the survival and/or functioning of the rhizobial partner (Moorman et al., 1992; Royuela et al; 1998; Prather et al., 2000; Zablotowicz and Reddy, 2004).

Singh and Wright (2002) studied the direct effect of photosynthetic inhibitor herbicides on the growth of *R. leguminosarum*. They performed an *in vitro* experiment to study the effects of terbutryn/terbuthylazine, trietazine/simazine, prometryn and bentazon on the growth of rhizobia in liquid media, which was estimated using optical density measurements. They determined that terbutryn/terbuthylazine significantly reduced growth of rhizobia at herbicide concentrations higher than 4.0 mg L^{-1}. Similarly, trietazine/simazine at concentrations higher than 4.3 mg L^{-1} significantly decreased rhizobial growth. Bentazon did not adversely affect rhizobial growth even at high concentration rates of 65.6 mg L^{-1}.

Drew et al. (2007) planted field pea and applied herbicides known to inhibit the ACCase enzyme. Inhibition of the ACCase enzyme slows down and stops fatty acid biosynthesis (Hall et al., 1999). In their experiment, two out of three of the ACCase inhibiting herbicides significantly reduced effective nodulation, %Ndfa and grain Ndfa. It is uncertain if the herbicides directly affected rhizobia; however, the reduction in effective nodulation suggested that the herbicides were interfering with the establishment of the legume-*Rhizobium* relationship. Santos et al. (2006) examined direct herbicidal effects of an ACCase inhibiting herbicide, fluazifop-p-butyl, on cell

growth of *R. tropici* and reported that cell growth was reduced

Growth regulators, such indole acetic acid (IAA), are involved in root nodule development (Dullaart et al., 1971). Today, synthetic auxins are used as herbicides to mimic IAA to kill target plants. Dullaart et al. (1971) examined the relationship between IAA and rhizobia. In their experiment, they inoculated *R. lupine* into a medium containing the synthetic auxin IAA. Initial growth of rhizobia was suppressed. However, when IAA was added to rhizobia during the logarithmic growth phase, growth was not inhibited. They concluded that only cells in the lag phase of growth were affected by the addition of the auxin IAA. This implies that the timing of the interaction between rhizobia and herbicides may be important in exhibiting negative effects.

In laboratory experiments, Fabra et al. (1997) found that 1 mM of 2,4-D negatively affected the growth of rhizobia. Moreover, their results are similar to those of Dullaart et al. (1971), who reported that the timing of the herbicide addition to rhizobia dictated the degree of the negative interactions. When 2,4-D was added to the culture in the stationary phase of incubation, the herbicidal effect on the rhizobia was exacerbated.

Herbicides whose mechanism of action is through the inhibition of amino acid biosynthesis (i.e., EPSPS and ALS/AHAS) may affect rhizobia because microbes also share this biosynthetic pathway (Burnet and Hodgson, 1991; Royuela et al., 1998; Prather et al., 2000; Zablotowicz and Reddy, 2004). An example is the herbicide glyphosate, which inhibits the enzyme EPSPS in the shikimic acid pathway. Rhizobia similarly possess this glyphosate-sensitive enzyme (Zablotowicz and Reddy, 2004). In greenhouse and field experiments, Zablotowicz and Reddy (2004) found that nodulation and nodule leghemoglobin content was inhibited by glyphosate applications on genetically modified soybean. Research by Eberbach and Douglas (1989) has also shown that glyphosate inhibits the nodulation ability of *R. trifolii*. While these results do not indicate a herbicidal affect on rhizobia, they do indicate that the herbicide may affect rhizobia directly by decreasing their ability to nodulate.

Other examples of herbicides that inhibit amino acid biosynthesis include those that target the enzyme acetolactate synthase. This enzyme is involved in synthesizing branched chain amino acids in both plants and rhizobia (Royuela et al., 1998). Sawicka and Selwet (1998) examined the effect of imazethapyr (an ALS inhibitor) on *Rhizobium*

in a field experiment in which they applied imazethapyr on pea, horse bean, yellow lupine, white lupine and soybean and measured N_2 fixation using ARA. In all post-emergent applications, imazethapyr significantly decreased nitrogenase activity. In order to determine direct negative interactions between rhizobia and ALS herbicides, Anderson et al. (2004) used an *in vitro* study to examine the growth of rhizobia in yeast mannitol broth (YMB) mixed with chlorosulfuron. Chlorosulfuron applied at the 2× field rate did not influence the growth of rhizobia.

Exploring the effects herbicides may have on soil microorganisms is very important, particularly as rhizobia are crucial in maintaining soil fertility by their ability to convert atmospheric N_2 to forms that are available to plants. Thus far, in the field and growth chamber experiments examining the impact of herbicide application on the legume-*Rhizobium* association, results demonstrated that the herbicides affected the plant and in the worst case scenario, the photosynthate supply to the roots and rhizobia. However, evidence suggests that overall N_2 fixation was unaffected. In the previous experiments, soil has been the media in which the microbes and the plants interacted. This final experiment was conducted to determine direct effects of the herbicides on rhizobia that were previously undetected because the soil acted as a natural buffer. In this experiment, rhizobia were grown in YMB and were directly mixed with residual herbicides known to have properties associated with auxins and ACCase inhibitors (clopyralid + MCPA, and clodinafop-propargyl) and ALS/AHAS inhibitors (flucarbazone-sodium).

5.2 Materials and Methods

5.2.1 Determination of herbicide rates

Determining the amount of herbicide that approximated rates that might occur in soil solution required several assumptions. First, it was assumed that the soil bulk density was 1.3g cm^{-3}. This infers that in a hectare furrow slice, there are 13 000 Mg of soil. Using the methodology of Fletcher (1956), it was also assumed that the soil moisture content was 20%. Therefore, in 13 000 Mg of soil, there would be 2.6 million kg, or liters, of soil water. The 2× recommended field rate for clopyralid + MCPA (Curtail M) is 198 g a.i. ha^{-1}. Therefore, there are 198 g a.i. for 2.6 million liters of soil

water, which equals 76 µg a.i. L^{-1} of soil water. The actual amount of clopyralid + MCPA added to the flask was 0.15 µL of solution 100 mL^{-1} in the flask for the 2× rate. All other herbicide amounts were similarly determined.

5.2.2 Establishing a growth curve

Before combining herbicides and rhizobia, a growth curve was developed to determine the natural growth rate of *R. leguminosarum*. Initially a YMB was prepared and pH was brought to 7 with 1N NaOH (Vincent, 1970). In preparation for the experiment, 100 mL were poured into 19 250-mL side-arm flasks and one Erlenmeyer flask. The flasks were autoclaved for 20 min on a liquid cycle at 121°C and 103 kPa 15 BAR. The Erlenmeyer flask was inoculated with 1 mL of *R. leguminosarum* (bivar viceae strain 128c stock, obtained from Nitragin). After inoculation, the flask was shaken and 0.1 mL was removed and plated on yeast mannitol agar (YMA) amended with Congo Red. Duplicate plates were prepared and the plates were then placed in a dark cupboard upside down at room temperature to determine the purity of the culture and the population of colony forming units. The flask was placed on a shaker (New Brunswick Scientific G10 Gyrotory) at 115 rpm at room temperature (25°C). The flask was shaken for 48 h and then removed. One milliliter of broth was aseptically removed from the flask and inoculated into each of the side-arm flasks. One of the side-arm flasks was swirled to ensure homogeneity and 0.1 mL was removed and plated (Time = 0). Duplicate plates were prepared. Afterwards, all side-arm flasks were measured for optical density on a KLETT Summerson Photoelectric Colorimeter. The colorimeter used filter #66 (Red) which reads an approximate spectral range of 640 to 700 µm (Klett Manual, 1969). An uninoculated vessel containing only YMB was used to zero the spectrophotometer. The replicate flasks allowed for duplicate destructive sampling.

Sampling included aseptically removing 1 mL of culture from the side-arm flask and inoculating a test tube containing 9 mL of autoclaved phosphate buffer (1.2g Na_2HPO_4, 0.18g NaH_2PO_4 and 8.5g NaCl per liter). The test tube was vortexed for homogeneity and 1 mL was removed and placed in a second test tube, again containing 9 mL of phosphate buffer. The number of dilutions depended on the time of sampling. Typically, dilutions ranged from 1 to 3 during early growth stages, and 7 to 8 dilutions during active and later growth stages. After dilutions were made, 0.1 mL was removed

from the test tube and plated on YMA with Congo Red. Plates were placed in a Fisher incubator at 28°C. Sampling and plating was replicated twice. Sampling times were 0, 8, 12, 16, 20, 20, 24, 28, 32, 36, 40, 48, 56, 64, 72, 80, 88, 96, 110 and 158 h after flask inoculation. Four days after sampling and plating, colony forming units were counted. The optical density and colony forming units were plotted (Fig 5.1) using Excel (2008). Optical density increased linearly until a plateau was reached. A linear regression equation was used to describe the linear portion of the relationship between optical density and colony forming units as follows:

$$\text{Colony forming units} = 7\times10^{9}x - 5\times10^{7} \qquad \text{Eq. [5.1]}$$

where x equals the optical density reading. For optical density readings exceeding the plateau, a population of 1.5×10^{9} was assumed.

5.2.3 Optical density and measurements

Side arm flasks were prepared by autoclaving 98 mL of YMB with a pH of 7.0 (Vincent, 1970). Herbicide solutions including clopyralid + MCPA, clodinafop-propargyl and flucarbazone-sodium were filter sterilized through 0.45 µm cellulose acetate membrane (VWR) then added to autoclaved distilled water. For the experiment, there was one control (autoclaved rhizobia) and four replicates of 0, 0.25, 0.5, 1.0, 1.5 and 2.0 × the recommended rates (for a total of 32 flasks per herbicide). The recommended field rate was equivalent to 99 g a.e. ha^{-1} + 554.4 g a.i. ha^{-1}, 55.7 g a.i. ha^{-1} and 28 g a.i. ha^{-1} for clopyralid +MCPA, clodinafop-proparygyl and flucarbazone-sodium, respectively. Each flask was aseptically inoculated with 1 mL of an active *Rhizobium* culture (grown in a mother culture for 3 days) and 1 mL of herbicide solution, with the exception of the blank that included autoclaved rhizobia. The flasks were placed on a rotary shaker at room temperature (25°C). The optical density was measured at 0 and 8 h and then every 4 h until 96 h. Additional samples were measured at 104, 112, 124, 128 and 161 h. Uninoculated vessels containing autoclaved rhizobia, YMB and the herbicide treatment were used as controls.

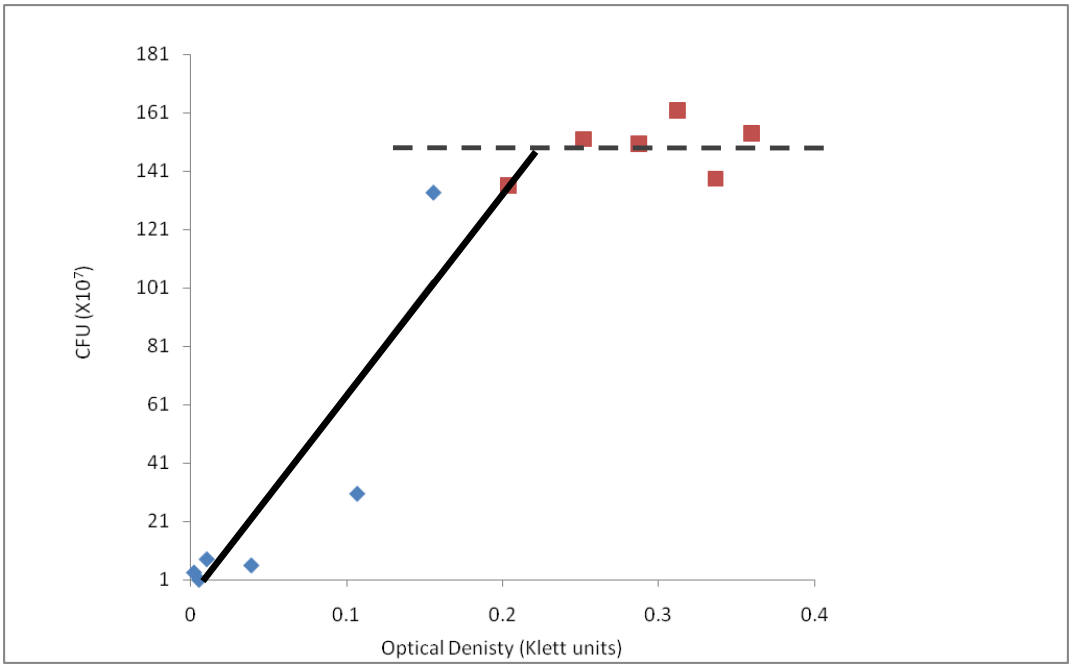

Figure 5.1 Relationship between optical density and colony forming units of *R. leguminosarum.*

5.3 Results and Discussion

Although data suggests a delay in rhizobial growth, differences were not statistically significant according to the ANOVA. There were no detrimental effects on survivial and growth of rhizobia due to treatments of clopyralid + MCPA mixed with clodinafop-propargyl or flucarbazone-sodium (Fig 5.2 and 5.3).

While previous research suggests that auxin herbicides have a direct negative effect on rhizobia (Dullaart et al., 1971; Fabra et al., 1997; Arias and Fabra de Peretti, 1993), the synthetic auxin herbicides clopyralid +MCPA, did not affect rhizobia when mixed with clodinafop-propargyl, an ACCase inhibitor. Clark and Mahanty (1991) grew rhizobia on agar plates with a center disk that had been dipped in fluzifop, an ACCase inhibitor. The 1× rate of herbicide caused a small zone of inhibition surrounding the disk, but the 10× rate had no zone of inhibition. When growing rhizobia in a broth culture, rhizobial growth was unaffected at both rates.

Other research has suggested that in cases where there was a herbicide effect on rhizobia, it was in situations where the herbicide rate far exceeded typical field application rates (Royuela et al., 1998; Singh and Wright, 2002). Royuela et al. (1998) studied the growth of rhizobia exposed to imazethapyr (an ALS/AHAS inhibitor) and concluded that there was no direct effect on rhizobia except when doses were 700× higher than the recommended field application rate. Similarly, Gonzalez et al. (1996) grew *R. leguminosarum* on pure culture yeast extract mannitol (YEM) with different concentrations of imazethapyr. After 7 d, there were no differences in rhizobial growth in treated and untreated cultures at concentrations between 0.34 mM to 3.4 mM. Results in this experiment using the herbicide flucarbazone-sodium (an ALS inhibitor), are similar to these studies in that there were no direct herbicidal effects on rhizobia, once active growth was established.

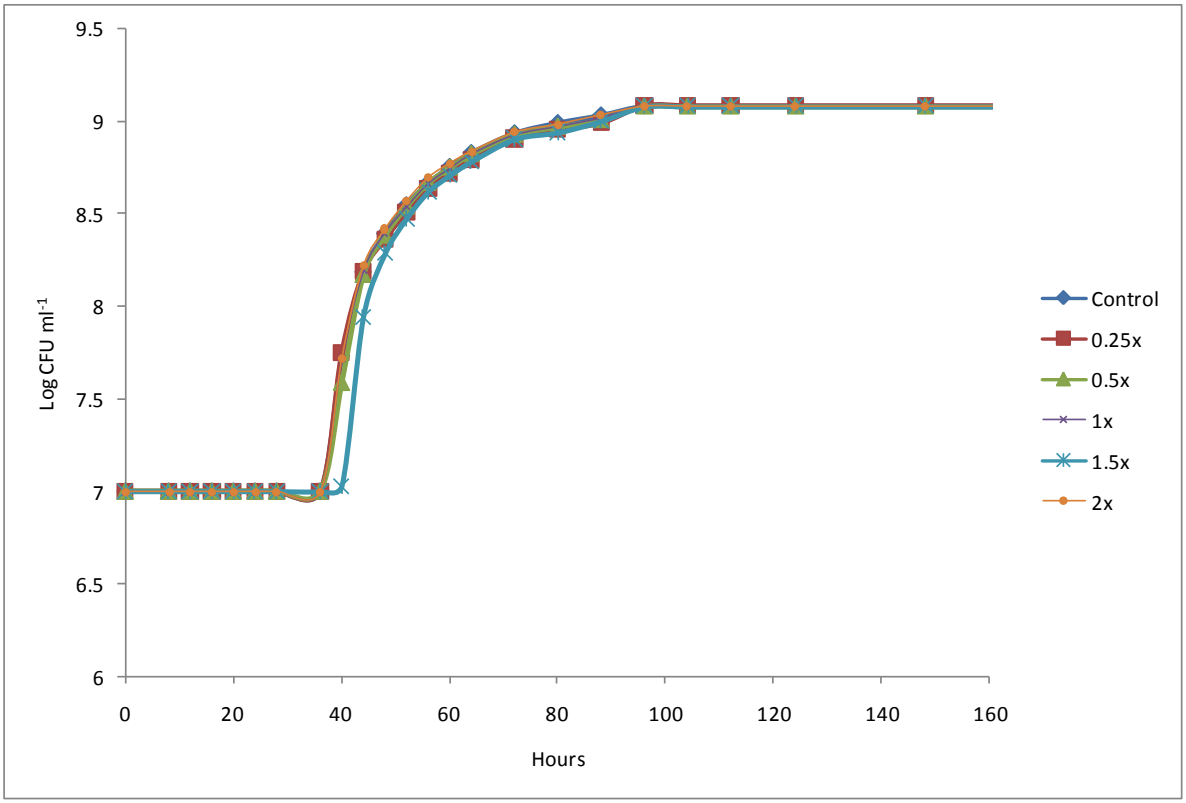

Figure 5.2 Colony forming units over time for rhizobia exposed to clopyralid + MCPA and clodinafop-propargyl.

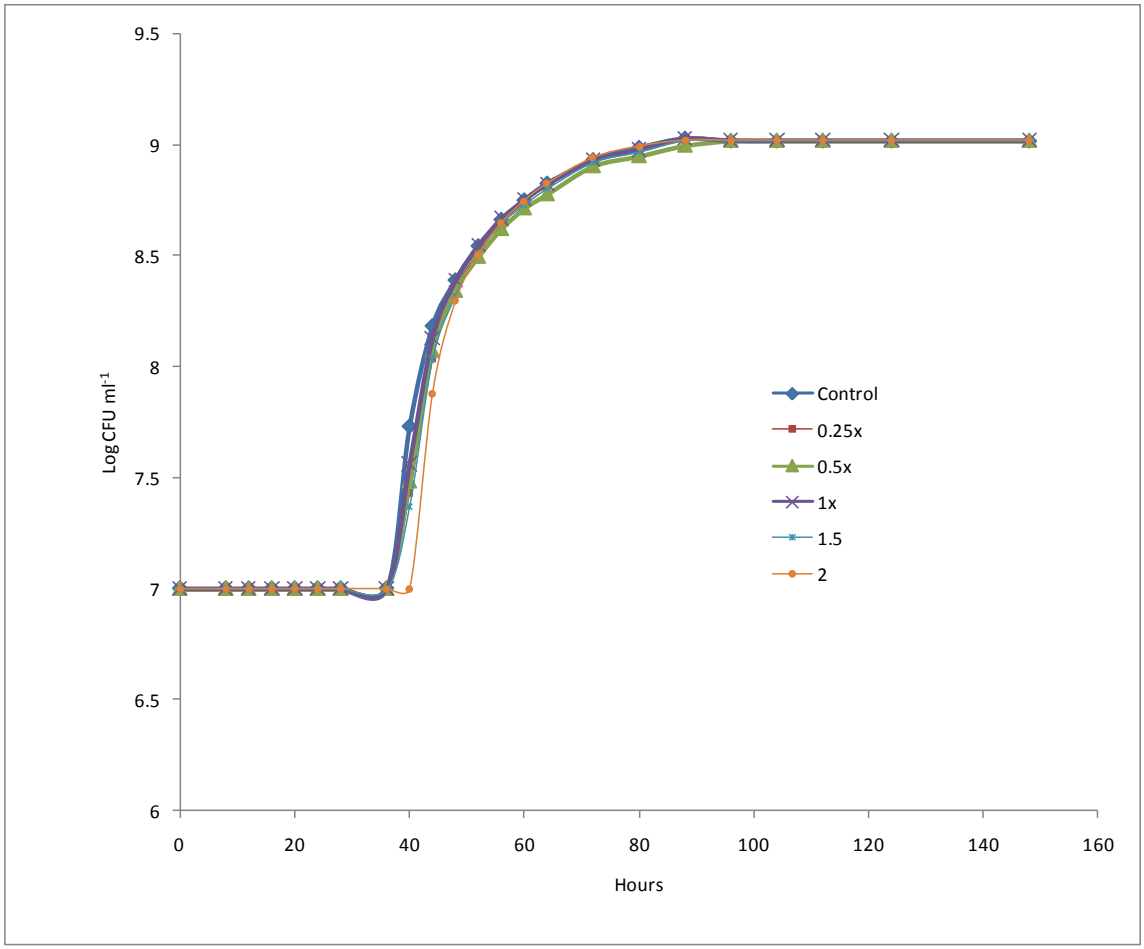

Figure 5.3 Colony forming units over time for rhizobia exposed to flucarbazone-sodium.

It is possible that conditions used in this experiment limited the impact of the herbicides on rhizobial growth. Yeast mannitol broth used in this experiment may have acted as a buffer between the rhizobia and the herbicides. Mårtensson (1992) stated that the presence of agar may influence the mode of action of the herbicides. Additionally, when using the broth method, there is the possiblilty of adsorption or precipitates between the herbicides and broth media components, although none were visually observed.

Lastly, some organisms may not be inhibited by the herbicides because they have a non-sensitive ALS enzyme (Burnet and Hodgson, 1991; Royuela et al., 1998). There are three different forms of the enzyme and each one has a different mode of regulation at the level of gene expression. Perhaps the rhizobia are not sensitive to the herbicide (Royuela et al., 1998).

5.4 General Conclusions

Herbicide application is a routine part of industrialized agriculture. The two experiments were designed to study direct effects of the herbicides clopyralid + MCPA (auxin) and clodinafop-propargyl (ACCase inhibitors) as well as the herbicide flucarbazone-sodium (ALS/AHAS inhibitors) and growth of rhizobia in YMB. The herbicides and *Rhizobium* were mixed together in a YMB and the growth of the rhizobia was monitored. Although early growth appeared to be limited by the herbicides used in these experiments, differences were not statistically significant. Even when the herbicides were applied at 2× field application rates, there were no observable changes in growth of rhizobia. In similar experiments, other authors have found limited herbicidal effects on the growth of rhizobia. Only in cases where the herbicide application rate far exceeded any recommended field rate did they find that rhizobial growth was affected (Royuela et al., 1998; Singh and Wright, 2002).

Whether the media interrupted natural interactions between rhizobia and the herbicides used in this study (Mårtensson, 1992) or the rhizobia were not sensitive to the enzyme inhibitor (Burnet and Hodgson, 199; Royuela et al., 1998), there were little or no discernable negative effects on the growth pattern of rhizobia. This observation supports the notion that negative interactions between these herbicides and N_2 fixation

are due largely to negative effects on the plants.

6 GENERAL SUMMARY AND CONCLUSIONS

The goal of this project was to determine if herbicides, commonly used in western Canada, negatively affect N_2 fixation in pulse crops and to identify possible mechanisms by which herbicides may influence N_2 fixation. In particular, there were three objectives:

1. To assess the impact of pre- and post-emergent herbicides used for weed control in field pea and chickpea on nodulation, N_2 fixation and consequent yield.

2. To assess the impact of herbicides, known to have residual properties and used in crops preceding field pea, on nodulation, N_2 fixation and consequent yield.

3. To compare the use of inoculation types (i.e., granular soil applied inoculant versus peat powder seed applied inoculant) on N_2 fixation by pea subject to herbicide stress and to determine if inoculation and type of inoculant influences the impact of herbicides on N_2 fixation.

The project included field experiments conducted over two field seasons to represent natural conditions, a growth chamber experiment to represent controlled conditions and lastly, a laboratory experiment to examine specific interactions between the herbicides and the rhizobia.

The legume-*Rhizobium* symbiotic relationship is dependent on a combination of soil type, fertility, crop, *Rhizobium* species, and environment. For example, if weather and soil conditions are favourable for the symbiotic relationship, but the native or inoculated *Rhizobium sp*. is ineffective, N_2 fixation will be limited. The additions of herbicides add an unknown dynamic into the legume-*Rhizobium* relationship and there is a possibility for decreased N_2 fixation.

Herbicides may affect N_2 fixation by several mechanisms. They can affect plant growth leading to a decreased photosynthate supply to roots and thus decrease the number of available infection sites for *Rhizobium* (Rennie and Dubetz, 1984; Bertholet and Clark, 1985; Sprout et al., 1992; Singh and Wright, 1999; Abd-Alla et al, 2000, Singh and Wright, 2002, Anderson et al., 2004). Additionally, if a plant is redirecting photosynthate to shoot biomass to facilitate regrowth following herbicide injury, less

photosynthate is available for rhizobial growth and survival.

Herbicides may also directly interfere with rhizobial function and growth (Moorman et al., 1992; Singh and Wright 1999; Singh and Wright, 2002; Anderson et al., 2004). Herbicides, particularly those that inhibit the enzyme ALS/AHAS and ACCase, can affect both plants and microbes if these organisms have similar enzyme pathways. Consequently, there is a potential for interference with cell metabolism in non-target organisms, such as rhizobia (Mårtensson, 1992; Singh and Wright, 1999; Musarrat and Haseeb, 2000; Singh and Wright, 2002, Fox et al., 2001; Anderson, et al., 2004 and Fox et al., 2004).

The first part of this project included a series of field experiments, which were carried out to examine the impact of herbicides on N_2 fixation under natural dryland conditions. Three separate experiments were conducted. The first experiment examined the impact of pre- and post-emergent herbicides on N_2 fixation in field pea. There were two locations; one at Goodale, SK and one at Clavet, SK. Although various effects on both plant growth and N_2 fixation were observed, these were inconsistent between the different sites and typically were limited to the early sampling times. For example, the only obvious negative trend found at both sites was herbicide damage observed at the early sampling time (7DAA). The damage included decreases in biomass, and NA at both sites. Additionally, at Clavet, herbicide effects included a reduction in nodule biomass. However, by the July sampling dates, there were no differences in these measurements, suggesting a complete recovery from the early effects on crop growth and N_2 fixation. It was concluded that the herbicides used in this experiment (including amitrole, glyphosate, MCPB + MCPA, MCPA, imazethapyr + imazamox, metribuzin and bentazon) did not directly affect N_2 fixation or final yield, irrespective of early herbicide damage symptoms.

The second field experiment examined the affect of pre- and post-emergent herbicide application on N_2 fixation in chickpea. This experiment was conducted at Goodale, SK. One of the interesting aspects of this experiment was that application of metribuzin, one of two products registered for chickpea (two of the products tested were not registered for use in Canada), consistently decreased shoot biomass (measured 7 DAA), NA (measured 29 DAA) and nodule biomass (measured both 7 and 29 DAA). Although,

parameters such as NA and nodule biomass were decreased by the metribuzin treatments, differences in N accumulation, specific NA, yield, protein, Ndfa, seed Ndfa and harvest index, were not statistically significant. According to data collected in this experiment, N_2 fixation was reduced 29 DAA by the application of metribuzin (1× and 2× field recommended rates), and sulfentrazone, but specific NA was unaffected and %Ndfa did not differ between these treatments. Therefore, in this cropping year, N_2 fixation and yield were not inhibited by the pre- and post- emergent applications of the herbicides metribuzin (1× and 2× field recommended rate), sulfentrazone, isoxaflutole and imazethapyr + glyphosate.

The third field experiment examined the effect of residual herbicides on N_2 fixation in field pea, together with the impact of inoculant formulation. The experiment was conducted at Goodale, SK. Treatments examining the impact of inoculating field pea with granular (soil applied) and peat (seed applied) inoculants were included to determine if inoculant type influenced interactions between the residual herbicides and N_2 fixation. Wheat was planted the previous year and herbicides with known residual properties (clopyralid, flucarbazone-sodium and sulfosulfuron) were applied to the crop. Field pea was planted the following year. This field experiment first took place in 2005 and was repeated in 2006. Over the period of this experiment, environmental conditions were conducive to microbial degradation of the herbicides and few N_2 fixation parameters were affected (%Ndfa and seed Ndfa decreased in pea planted in clopyralid + MCPA and clodinafop-propargyl residues in 2005, but not 2006). It was concluded that little herbicide residue was carried over from the previous year and thus, no overall negative interactions between the herbicides and N_2 fixation were observed. Yield decreased in 2006 in pea planted in flucarbazone-sodium residues (20 g a.i. and 30 g a.i.), but again, there were no differences in early season measurements and the decrease in yield is not associated with an interaction between pea and herbicide residue.

The second part of this field experiment was to determine if inoculant formulation, e.g., granular (soil applied) or peat (seed applied), mitigated possible effects on negative interactions between N_2 fixation and yield. Peat-inoculated pea plants had increased NA and specific NA, but only in 2005. There were no differences in %Ndfa or seed Ndfa, therefore the mid-season measurements may not have been indicative of the overall

season N_2 fixation. It was concluded that inoculant formulation (granular versus peat) influence interactions between residual herbicides including clopyralid + MCPA and clodinafop-propargyl, flucarbazone-sodium and sulfosulfuron.

A second component of this project examined the impact of residual herbicides on N_2 fixation of field pea under controlled growth chamber conditions. In addition to application of herbicides at varying rates, field pea was grown with and without inoculant to examine any differences between the native and the inoculated rhizobial populations. In the field experiment, the amount of residual herbicide carried over was unknown. In fact, it was concluded that little, if any herbicide residue was carried over as microbes likely degraded the herbicide due to the favorable environmental conditions. The growth chamber experiment was designed to study a range of residual herbicide levels and did not mimic natural degradation in a field. Specifically, no degradation products were considered.

The first experiment included the tank mix of clopyralid + MCPA and clodinafop-propargyl, while the second experiment used flucarbazone-sodium. In both experiments, reductions in shoot biomass, nodule mass and nodule number were observed only where herbicides were applied at 1× the field recommended rate – a rate intended to simulate a situation where no degradation of the herbicide occurred following application in the previous year. Interestingly, although some growth parameters were negatively affected by the highest herbicide application rate, specific NA was enhanced at this application rate. This led to the conclusion that although these herbicides may negatively affect biomass and nodulation, once rhizobia penetrate the root, their ability to fix N_2 is unaffected and, in some cases stimulated. It is important to note, however, that it was only plants exposed to the 1× application rate that were severely damaged and it is unlikely that these high levels of residual herbicide would be encountered under normal field conditions.

Plants in the worst case scenario (e.g., 1× field recommended rate) were visibly physically damaged, but the ability of the rhizobia to fix N_2 was unaffected. This led to the conclusion that clopyralid + MCPA and clodinafop-propargyl and flucarbazone-sodium, may affect plant growth, but they do not inhibit N_2 fixation directly.

The third part of this project examined direct herbicidal effects on rhizobial growth.

In the field experiments and growth chamber experiments, soil acted as a buffer between the herbicides and the rhizobia. Whereas it seemed that only plant growth was affected directly, the survival of rhizobia in direct contact with the herbicides was not monitored.

The lab experiment was conducted to further investigate the potential effect of herbicides on rhizobial growth and survival. The *in vitro* experiments were performed to observe the growth of rhizobia in direct contact with a of range herbicides without soil as a buffer. The experiments used the same herbicides as were used in the field and growth chamber studies (e.g., clopyralid + MCPA and clodinafop-propargyl and flucarbazone-sodium at rates equivalent to a range from 0× to 2× the recommended field application rate). For both herbicide treatments, any differences in growth due to direct contact between the rhizobia and the herbicides were not significant. It is not known if rhizobia were capable of degrading the herbicides and using them as a source of C, or if the herbicides precipitated or adsorbed in the YMB, but solely based on observations, there was no detectable effect of these herbicides on rhizobial growth.

Further studies examining the relationship between herbicides and N_2 fixation are warranted. The majority of research examining herbicide and N_2 fixation interaction has been conducted in the United States, Australia and Europe with very little research focused on crops grown in conjunction with herbicides used in western Canada. While this project began with a broad spectrum of herbicides (pre- and post-emergent as well as residual herbicides), it was narrowed to residual herbicides in the growth chambers and laboratory experiments. Because of the value of the legume-*Rhizobium* relationship to supply plants with N as well as increase soil N reserves, additional research is warranted to study the effects of other herbicides on rhizobia and its ability to fix N_2, particularly herbicides that inhibit enzyme pathways that also exist in both the target plant and the rhizobia.

This project has focused on herbicide interactions in field pea, with only one field experiment using chickpea. As pulse crops, including lentil, chickpea and soybean, are becoming a greater part of crop rotations, further research ought to be conducted to examine possible negative effects of herbicides on N_2 fixation in these crops.

Nitrogen fixation associated with the legume-*Rhizobium* relationship offers benefits not only to the legumes themselves, but potentially to subsequent crops by increasing the

amount of soil N. This in turn, decreases fertilizer costs. Herbicides that are suitable for agricultural use should have very little to no effect on N_2 fixation in order to be considered for use in a pulse crop rotation. This project examined the impact of a variety of herbicides on the legume-*Rhizobium* relationship at the microbial level, in a controlled growth chamber environment and under field conditions. Limited to the herbicides used in this project, N_2 fixation was unaffected and growers may be assured that the application of these herbicides at field recommended rates, will not interfere with N_2 fixation.

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8 APPENDIX

Appendix A: ANOVA tables for data collected from field sites in Saskatchewan (2005 and 2006), as well as data collected from a growth chamber experiment (2005).

Table A.1 Herbicides used for field studies.

Chemical name	Trade name	Application timing	Formulation	Concentration	Manufacturer/Distributor
				g a.i.L^{-1} †	
Amitrole	Amitrol 240	Pre-emerg.	Solution	231	Nufarm
Bentazon	Basagran	Post-emerg.	Solution	480	BASF
Bromoxynil + MCPA	Buctril M	Post-emerg.	EC	280 + 280	Bayer CropScience
Clodinafop-propargyl	Horizon	Post-emerg.	EC	240	Syngenta
Clopyralid + MCPA	Curtail M	Post-emerg.	EC	50 + 280	Dow AgroSciences
Flucarbazone-sodium	Everest	Post-emerg.	Water soluble packets	70†	Arysta Lifescience
Glyphosate	Round-Up Transorb	Pre-emerg.	Solution	540	Monsanto
Imazamox + imazethapyr	Odyssey	Post-emerg.	Dispersable granule	35† + 35†	BASF
Imazethapyr	Pursuit	Post-emerg.	Solution	240	BASF
Isoxaflutole	Converge Pro	Pre-emerg.	Soluble concentrate	480	Bayer CropScience
Metribuzin	Sencor 75DF	Post-emerg.	Dispersable granule	75†	Bayer CropScience
MCPB + MCPA	Tropotox - Plus	Post-emerg.	Solution	375 +25	Nufarm
Sulfentrazone	Spartan	Pre-emerg.	Dry flowable	75†	FMC Corporation
Sulfosulfuron	Sundance	Post-emerg.	Dispersable granule	75†	Monsanto

† Denotes percentage.

Table A.2 ANOVA for plant biomass and N accumulation response to pre- and post- emergent herbicide application to field pea (Experiment 1) at Clavet, SK (2005).

	Shoot Biomass				Nodule Biomass				N accumulation			
					June							
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	12	0.089	3.752	0.001	12	0.000	3.737	0.001	12	2.396	4.034	0.001
Block	5	0.067	2.841	0.030	5	0.000	4.406	0.003	5	1.823	3.068	0.021
Herbicide	7	0.104	4.403	0.001	7	0.000	3.260	0.009	7	2.806	4.724	0.001
Error	35	0.024			35	5.03E-005			35	0.594		
					July							
Model	12	1.709	1.671	0.117	12	0.050	3.260	0.003	12	23.927	1.183	0.332
Block	5	2.686	2.627	0.041	5	0.051	3.287	0.015	5	43.968	2.174	0.079
Herbicide	7	1.012	0.989	0.455	7	0.050	3.240	0.009	7	9.612	0.475	0.846
Error	35	1.023			35	0.015			35	20.223		

Table A.3 ANOVA for NA and specific NA response to pre- and post- emergent herbicide application to field pea (Experiment 1) at Clavet, SK (2005).

	NA†				Specific NA g^{-1} nodule			
	June							
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	12	2 055	4.882	0.000	12	1 092 976	2.370	0.023
Block	5	2 602	6.180	0.000	5	181 569	0.394	0.850
Herbicide	7	1 664	3.954	0.003	7	1 743 981	3.782	0.004
Error	35	421			35	461 088		
	July							
Model	12	83 691	2.159	0.038	12	299 300	0.960	0.503
Block	5	94 955	2.450	0.053	5	645 063	2.069	0.093
Herbicide	7	75 645	1.952	0.091	7	52 326	0.168	0.990
Error	35	38 761			35	311 778		

† NA = Nitrogenase activity

Table A.4 ANOVA for pre- and post-emergent herbicide effects on yield, protein, %Ndfa, seed Ndfa and harvest index in field pea (Experiment 1) at Clavet, SK (2005).

	Yield				Protein				%Ndfa			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	12	65 278 897	4.106	0.001	12	1.969	1.665	0.118	12	275.138	1.124	0.373
Block	5	109 047 668	6.859	0.000	5	23 427	19 802	0.000	5	348.182	1.423	0.240
Herbicide	7	34 015 489	2.139	0.065	7	1.406	1.189	0.335	7	222.964	0.911	0.510
Error	35	15 899 222			35	1.183			35	244.761		

	Seed Ndfa				Harvest index			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	12	382.041	1.224	0.306	12	0.000	0.578	0.845
Block	5	435.731	1.397	0.250	5	0.001	0.848	0.525
Herbicide	7	343.691	1.102	0.384	7	0.000	0.385	0.905
Error	35	312.002			35	0.001		

Table A.5 ANOVA for plant biomass and N accumulation response to pre- and post- emergent herbicide application to field pea (Experiment 1) at Goodale, SK (2005).

	Shoot Biomass				Nodule Biomass				N accumulation			
					June							
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	10	0.393	2.251	0.056	10	0.007	5.138	0.001	10	6.900	23.436	0.041
Block	3	0.338	1.938	0.154	3	0.013	10.224	0.000	3	10.248	3.617	0.030
Herbicide	7	0.416	2.386	0.058	7	0.004	2.958	0.025	7	5.465	1.929	0.115
Error	21	0.174			21	0.001			21	2.833		
					July							
Model	10†	0.028	2.877	0.020	10	0.089	1.399	0.247	10	296.320	2.187	0.063
Block	3	0.014	1.406	0.269	3	0.164	2.576	0.081	3	266.436	1.966	0.150
Herbicide	7	0.034	3.507	0.012	7	0.057	0.894	0.529	7	309.128	2.281	0.068
Error	21	0.010			21	0.064			21	135.501		

† indicates July shoot biomass transformed by log function

Table A.6 ANOVA for NA and specific NA response to pre- and post- emergent herbicide application to field pea (Experiment 1) at Goodale, SK (2005).

	NA†				Specific NA g^{-1} nodule			
				June				
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	10	50 717	4.734	0.001	10	1 046 771	197 198 340	0.000
Block	3	89 876	8.389	0.001	3	1 074 479	8.722	0.001
Herbicide	7	33 934	3.167	0.019	7	1 034 895	8.400	0.000
Error	21	10 713			21	123 196		
				July				
Model	10	111 829	0.621	0.780	10	323 123	1.464	0.221
Block	3	41 456	0.230	0.874	3	662 539	3.001	0.054
Herbicide	7	141 989	0.788	0.605	7	177 660	0.805	0.593
Error	21	180 092			21	220 784		

† NA = Nitrogenase activity

Table A.7 ANOVA for pre- and post-emergent herbicide effects on yield, protein, %Ndfa, seed Ndfa and harvest index in field pea (Experiment 1) at Goodale, SK (2005).

	Yield				Protein				%Ndfa			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	10	1 432 721	1.030	0.453	10	1.758	2.084	0.075	10	2 424.791	3.938	0.004
Block	3	2 931 917	2.108	0.130	3	1.567	1.858	0.168	3	6 678.242	10.845	0.000
Herbicide	7	790 208	0.568	0.773	7	1.840	2.81	0.079	7	601.883	0.977	0.473
Error	21	1 391 155			21	0.844			21	615.807		

	Seed Ndfa†				Harvest index†			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	10	0.724	1.797	0.124	10	0.040	0.857	0.583
Block	3	1.420	3.523	0.033	3	0.035	0.758	0.530
Herbicide	7	0.426	1.057	0.424	7	0.042	0.900	0.524
Error	21	0.403			21	0.047		

† indicates data transformed by log function

Table A.8 ANOVA for plant biomass and N accumulation response to pre- and post- emergent herbicide application to chickpea (Experiment 2) at Goodale, SK (2005).

	Shoot Biomass				Nodule Biomass				N accumulation			
					June							
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	8	0.322	9.944	0.000	8	0.001	2.320	0.076	8	1.314	2.984	0.032
Block	3	0.087	2.695	0.083	3	0.000	1.332	0.301	3	1.092	2.480	0.101
Herbicide	5	0.462	14.294	0.000	5	0.001	2.912	0.049	5	1.447	3.286	0.033
Error	15	0.032			15	0.000			15	0.440		
					July							
Model	8	3.937	2.703	0.046	8	0.037	4.132	0.009	8	24.773	1.777	0.161
Block	3	0.964	0.662	0.588	3	2.201	248.364	0.000	3	14.586	1.046	0.401
Herbicide	5	5.720	3.927	0.018	5	0.035	3.927	0.018	5	30.886	2.215	0.107
Error	15	1.456			15	0.009			15	13.945		

Table A.9 ANOVA for NA and specific NA response to pre- and post- emergent herbicide application to chickpea (Experiment 2) at Goodale, SK (2005).

	NA†				Specific NA g^{-1} nodule			
	June							
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	8	570.725	1.566	0.216	8	501 134	2.728	0.045
Block	3	688.575	1.890	0.175	3	749 657	4.081	0.026
Herbicide	5	500.016	1.372	0.289	5	352 020	1.916	0.151
Error	15	364.345			15	183 712		
	July							
Model	8	37 199	4.085	0.009	8	115 263	1.780	0.160
Block	3	24 578	2.699	0.083	3	135 197	2.088	0.145
Herbicide	5	44 772	4.917	0.007	5	103 302	1.596	0.221
Error	15	9 106			15	64 744		

† NA = Nitrogenase activity

Table A.10 ANOVA for pre- and post-emergent herbicide effects on yield, protein, %Ndfa, seed Ndfa and harvest index in chickpea (Experiment 2) at Goodale, SK (2005).

	Yield				Protein				%Ndfa			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	8	897 411	1.634	0.196	8†	0.001	1.060	0.438	8	1 510.268	1.341	0.297
Block	3	1 115 288	2.031	0.153	3	0.000	0.222	0.880	3	3 605.840	3.203	0.054
Herbicide	5	76 685	1.396	0.281	5	0.002	1.563	0.230	5	252.954	0.225	0.946
Error	15	549 119			15	0.001			15	1 125.887		

	Seed Ndfa†				Harvest index†			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	8	0.644	1.692	0.181	8	0.02	1.439	0.259
Block	3	1.014	2.664	0.086	3	8.011	572.173	0.000
Herbicide	5	0.422	1.109	0.396	5	0.014	0.994	0.454
Error	15	0.380			15	0.014		

† indicates data transformed by log function

Table A.11 ANOVA for shoot biomass, N accumulation, NA, nodule biomass and specific NA response to residual herbicide in field pea (Experiment 3) at Goodale, SK (2005).

	Shoot biomass				N accumulation				NA†			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	24	0.0938	1.60	0.1734	24	2.3259	1.57	0.1825	24	31 937	3.76	0.0052
Block	3	0.0343	0.34	0.7936	3	3.0731	1.78	0.2044	3	56 753	1.41	0.2866
Herbicide (main)	4	0.0393	0.39	0.8088	4	2.0834	1.21	0.3578	4	9 103	0.23	0.9180
Error (main)	12	0.0996	1.70	0.1648	12	1.726	1.17	0.382	12	40 110	4.72	0.0030
Inoculant (split)	1	0.0664	1.13	0.3038	1	5.8178	3.94	0.0658	1	71 008	8.36	0.0112
Herbicide × Inoculant	4	0.1826	3.12	0.0471	4	2.9344	1.99	0.1485	4	1 873	0.22	0.9227
Error (split)	15	0.0586			15	1.4776			15	8491		
	Nodule biomass				Specific NA							
Source of Variation	24	0.0049	4.12	0.0032	24	356 442	3.54	0.0070				
Block	3	0.0092	1.69	0.2211	3	449 969	2.07	0.1580				
Herbicide (main)	4	0.0050	0.93	0.4785	4	685 555	3.15	0.0548				
Error (main)	12	0.0054	4.58	0.0035	12	217 550	2.16	0.0802				
Inoculant (split)	1	0.0010	0.88	0.3618	1	1 173 121	11.65	0.0039				
Herbicide × Inoculant	4	0.0008	0.68	0.6194	4	169 693	1.69	0.2056				
Error (split)	15	0.0012			15	100 704						

† NA = Nitrogenase activity

Table A.12 ANOVA for residual herbicide effects on yield, protein, %Ndfa, seed Ndfa and harvest index in field pea (Experiment 3) at Goodale, SK (2005).

	Yield				Protein				%Ndfa			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	24	1 014649	1.96	0.0902	24	3.2001	1.94	0.0925	24	192.18	1.16	0.3926
Block	3	150 373	0.15	0.9275	3	10.3428	3.22	0.0612	3	885.79	14.51	0.0003
Herbicide (main)	4	1 963 444	1.96	0.1646	4	0.7497	0.23	0.9141	4	236.37	3.87	0.0303
Error (main)	12	1 000 252	1.93	0.1149	12	3.2086	1.95	0.1115	12	61.04	0.37	0.9562
Inoculant (split)	1	74 601	0.14	0.7099	1	3.5569	2.16	0.1624	1	0.85	0.01	0.9439
Herbicide × Inoculant	4	992 262	1.91	0.1606	4	0.1791	0.11	0.9776	4	69.08	0.42	0.7944
Error (split)	15	518 800			15	1.6478			15	166.01		
	Seed Ndfa				Harvest index							
Source of Variation	24	2 378	1.87	0.1053	24	0.0200	2.47	0.0367				
Block	3	5 831	3.76	0.0411	3	0.0203	0.98	0.4333				
Herbicide (main)	4	3 583	2.31	0.1175	4	0.0256	1.24	0.3458				
Error (main)	12	1 552	1.22	0.3524	12	0.0206	2.54	0.0454				
Inoculant (split)	1	66	0.05	0.8222	1	0.000	0.00	0.9649				
Herbicide × Inoculant	4	1 643	1.29	0.3170	4	0.0175	2.16	0.1230				
Error (split)	15	1 271			15	0.0081						

Table A.13. ANOVA for shoot biomass, N accumulation, NA, nodule biomass and specific NA response to residual herbicide in field pea (Experiment 3) at Goodale, SK (2006).

	Shoot biomass				N accumulation				NA†			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	24	7.98	0.95	0.5556	24	79.61	0.79	0.7065	24	23 270	1.13	0.4126
Block	3	13.45	1.65	0.2294	3	105.35	1.31	0.3154	3	49 700	2.07	0.1583
Herbicide (main)	4	3.31	0.41	0.8005	4	44.60	0.56	0.6987	4	17 987	0.75	0.5780
Error (main)	12	8.13	0.97	0.5137	12	80.20	0.79	0.6515	12	24 055	1.17	0.3825
Inoculant (split)	1	1.71	0.20	0.6576	1	18.69	0.19	0.6731	1	16 694	0.81	0.3823
Herbicide × Inoculant	4	9.69	1.16	0.3687	4	108.78	1.08	0.4021	4	8 021	0.39	0.8130
Error (split)	15	8.38			15	100.92			15	20 602		

	Nodule biomass				Specific NA			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*
Model	24	0.0117	4.99	0.0011	24	1 789832	1.41	0.2475
Block	3	0.0590	9.46	0.0017	3	2 833 555	1.48	0.2689
Herbicide (main)	4	0.0039	0.62	0.6544	4	1 288 214	0.67	0.6226
Error (main)	12	0.0062	2.65	0.0386	12	1 911 066	1.51	0.2243
Inoculant (split)	1	0.0008	0.36	0.5581	1	1 416 985	1.12	0.3073
Herbicide × Inoculant	4	0.0033	1.42	0.2768	4	1 238 171	0.98	0.4497
Error (split)	15	0.0023			15	1 268 749		

† NA = Nitrogenase activity

Table A.14 ANOVA for residual herbicide effects on yield, protein, %Ndfa, seed Ndfa and harvest index in field pea (Experiment 3) at Goodale, SK (2005).

	Yield				Protein				Ndfa			
Source of Variation	df	MS	F	*P*	df	MS	F	*P*	df	MS	F	*P*
Model	24	564 573	2.03	0.0794	24	1.4309	1.78	0.1237	24	71.49	4.29	0.0026
Block	3	839 835	1.99	0.1694	3	2.5478	2.49	0.1101	3	101.74	1.16	0.3666
Herbicide (main)	4	919 133	2.18	0.1333	4	3.1758	3.10	0.0572	4	70.74	0.80	0.5458
Error (main)	12	422 114	1.51	0.2212	12	1.0234	1.28	0.3235	12	88.00	5.29	0.0017
Inoculant (split)	1	425 414	1.53	0.2356	1	0.1441	0.18	0.6778	1	22.15	1.33	0.2668
Herbicide × Inoculant	4	465733	1.67	0.2087	4	0.3921	0.49	0.7441	4	12.35	0.74	0.5781
Error (split)	15	278 629			15	0.8024			15	16.65		
	Seed Ndfa				Harvest index							
Source of Variation	24	795.67	1.70	0.1444	24	0.0020	2.36	0.0445				
Block	3	472.39	0.53	0.6727	3	0.0020	1.04	0.4090				
Herbicide (main)	4	704.58	0.78	0.5567	4	0.0028	1.46	0.2755				
Error (main)	12	898.13	1.92	0.1166	12	0.0020	2.27	0.0678				
Inoculant (split)	1	1 124.44	2.40	0.1420	1	0.0033	3.92	0.0663				
Herbicide × Inoculant	4	739.68	1.58	0.2307	4	0.0011	1.25	0.3306				
Error (split)	15	468.12			15	0.0008						

Table A.15 Biomass and plant N means for field pea grown under controlled conditions in soil spiked with clopyralid + MCPA and clodinafop-propargyl.

Herbicide rate	Inoculant	Shoot biomass†	Shoot N concentration	Root biomass	Root N concentration
		g	%	mg	%
		Herbicide × inoculant means			
1×	Inoculated	0.9	3.61	167.5	3.80
	Uninoculated	0.8	3.78	205.0	2.80
0.5×	Inoculated	4.0	2.36	580.0	3.32
	Uninoculated	3.8	2.60	502.5	3.04
0.25×	Inoculated	4.3	2.93	750.0	3.22
	Uninoculated	4.3	2.75	700.0	2.96
0.125×	Inoculated	4.9	2.81	675.0	3.20
	Uninoculated	4.3	2.80	740.0	2.75
0.0625×	Inoculated	4.6	3.22	862.5	2.81
	Uninoculated	4.7	2.53	505.0	2.69
Control	Inoculated	4.5	2.64	404.3	3.21
	Uninoculated	4.2	2.55	677.5	3.37
		Herbicide means			
1×		0.9c*	3.70a	186.3c	3.30a
0.5×		3.9b	2.48c	541.3b	3.18ab
0.25×		4.3a	2.84b	725.0a	3.09ab
0.125		4.3a	2.80bc	707.5a	2.98bc
0.0625×		4.6a	2.88b	683.8a	2.75c
Control		4.6a	2.55bc	540.9b	3.29a
$LSD_{0.05}$		0.4	0.36	122.8	0.27
		Inoculant means			
	Inoculated	3.9a	2.91	573.2	3.26a
	Uninoculated	3.7b	2.93	555.0	2.93b
$LSD_{0.05}$		0.2	NS‡	NS	0.15

*Means followed by the same letter within each column are not significantly different according to the LSD ($P \leq 0.05$)

†denotes data based on two plants

‡ NS = not significant

Table A.16 Nodule mass, nodule number, NA and specific NA means for field pea grown under controlled conditions in soil spiked with clopyralid + MCAP and clodinafop-propargyl.

Herbicide rate	Inoculant	Nodule Mass†	NA	Specific NA
		mg	µmol ethylene h^{-1}	µmol ethylene g^{-1} nodule h^{-1}
			Herbicide × inoculant means	
1×	Inoculated	9.6	22	
	Uninoculated	25.3	20	2298
0.5×	Inoculated	410.3	354	3552
	Uninoculated	337.9	194	888
0.25×	Inoculated	522.6	308	558
	Uninoculated	403.0	224	596
0.125×	Inoculated	531.4	426	660
	Uninoculated	386.7	188	874
0.0625×	Inoculated	633.1	456	524
	Uninoculated	421.0	270	732
Control	Inoculated	307.3	270	640
	Uninoculated	256.8	270	624
			Herbicide means	
1×		174.5d*	20b	2926a
0.5×		374.1bc	274a	722b
0.25×		462.8ab	324a	628b
0.125		459.0ab	324a	698b
0.0625×		527.a	364a	686b
Control		282.0c	324a	758b
$LSD_{0.05}$		98.7	106	1330
			Inoculant means	
	Inoculated	396.3a	308a	1164
	Uninoculated	311.2b	198b	976
$LSD_{0.05}$		57.0	60	NS

Table A.17 Biomass and plant N means for field pea grown under controlled conditions in soil spiked with flucarbazone-sodium

Herbicide rate	Inoculant	Shoot biomass†	Shoot N concentration	Root biomass	Root N concentration
		g	%	mg	%
			Herbicide × inoculant means		
1×	Inoculated	2.0	3.88	580	3.28
	Uninoculated	2.2	3.61	418	3.23
0.5×	Inoculated	3.5	3.46	838	2.79
	Uninoculated	3.2	3.05	468	3.05
0.25×	Inoculated	4.8	2.73	778	2.95
	Uninoculated	5.0	2.33	623	3.04
0.125×	Inoculated	4.8	2.36	675	3.09
	Uninoculated	5.3	2.53	610	3.07
0.0625×	Inoculated	4.3	2.45	728	3.33
	Uninoculated	4.9	2.79	63	3.26
Control	Inoculated	4.5	2.64	528	3.17
	Uninoculated	4.2	2.55	640	3.40
			Herbicide means		
1×		2.1d*	3.75a	498b	3.14ab
0.5×		3.4c	3.25a	652a	2.92b
0.25×		4.9ab	2.53b	700a	3.00bc
0.125		5.0a	2.45b	643a	3.08abc
0.0625×		4.6ab	2.62b	683a	3.30a
Control		4.3b	2.55b	583ab	3.29a
$LSD_{0.05}$		0.6	0.54	134	0.27
			Inoculant means		
	Inoculated	4.1	2.91	681a	3.10
	Uninoculated	4.0	2.81	572b	3.16
$LSD_{0.05}$		NS‡	NS	77	NS

*Means followed by the same letter within each column are not significantly different according to the LSD ($P \leq 0.05$)
†denotes data based on two plants
‡ NS = not significant

Table A.18 Nodule mass, nodule number, NA and specific NA means for field pea grown under controlled conditions in soil spiked with flucarbazone-sodium.

Herbicide rate	Inoculant	Nodule Mass†	NA	Specific NA
		mg	µmol ethylene h^{-1}	µmol ethylene g^{-1} nodule h^{-1}
			Herbicide × inoculant means	
1×	Inoculated	151.7	276	1798
	Uninoculated	156.8	238	1566
0.5×	Inoculated	363.0	450	1234
	Uninoculated	319.0	446	1420
0.25×	Inoculated	434.2	450	1030
	Uninoculated	449.0	324	720
0.125×	Inoculated	473.6	328	684
	Uninoculated	526.3	328	624
0.0625×	Inoculated	397.1	440	1196
	Uninoculated	450.1	292	688
Control	Inoculated	307.3	270	622
	Uninoculated	256.8	270	302
			Herbicide means	
1×		154.2d*	256c	1682a
0.5×		341.0bc	448a	1328b
0.25×		441.6ab	386ab	874c
0.125		500.0a	328abc	654c
0.0625×		423.6ab	366abc	942c
Control		282.0c	270bc	462d
$LSD_{0.05}$		108.9	126	320
			Inoculant means	
	Inoculated	348.4	402	1068
	Uninoculated	365.8	410	912
$LSD_{0.05}$		NS	NS	NS

* Means followed by same letters within each column are not different according to the LSD ($P \leq 0.05$)

†denotes data based on two plants

‡ NS = not significant