INFLUENCE OF RESIDUAL FLUCARBAZONE-SODIUM ON INOCULATION SUCCESS MEASURED BY GROWTH PARAMETERS, NITROGEN FIXATION, AND NODULE OCCUPANCY OF FIELD PEA

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ABSTRACT

Herbicides have become a key component in modern agricultural production. Meanwhile, there is a concern that some herbicides persist past the growing season of the treated crop, and negatively influence the production of the subsequently planted crops. Amongst various herbicides used in western Canada, acetohydroxyacid synthase (AHAS)-inhibiting herbicides warrant special attention given their residual properties and acute plant toxicity at low concentrations in soil. Soil residual AHAS inhibitors have the potential to influence both leguminous host plants and their bacterial symbiotic partners; consequently, the use of an AHAS inhibitor in a given year can negatively influence the inoculation success and grain yield of legumes cropped in the following year. The present thesis project focused on one of the AHAS inhibiting herbicides (flucarbazone) and studied its potential for carryover injury and negative influence on the success of inoculation in field pea. A series of growth chamber and field experiments were conducted to test the following null hypothesis: *the presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia*.

A growth chamber experiment clearly demonstrated the susceptibility of field pea to the presence of flucarbazone in soil where the lowest concentration of flucarbazone amendment (5 $\mu g\ kg^{-1}$) significantly reduced the crop growth. In contrast, a field study failed to reveal any negative effects of flucarbazone use on crop growth and N_2 fixation. It was concluded that if the weather and soil conditions favour decomposition of flucarbazone as described in the present study, flucarbazone applied at the recommended field rate will not persist into the following season at high enough concentrations to negatively influence field pea growth, grain yields, and inoculation success. To ensure safety of rotational crops, it is important to strictly adhere to the herbicide application guidelines. Additionally, producers are cautioned to be particularly aware of the environmental and soil conditions that may reduce the rate of herbicide degradation.

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LIST OF ABBREVIATIONS

AHAS	Acetohydroxyacid synthase
ALS	Acetolactate synthase
a.i. ha^{-1}	Active ingredient per hectare
AB	Alberta
ATCC	American Type Culture Collection
BLAST	Basic local alignment search tool
bp	Base-pair
bv	Biovar
C	Cytosine
cfu	Colony-forming unit
DAS	Days after seeding
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
ED	Euclidian distance
ELISA	Enzyme-linked immuno sorbent assay
EPA	Environmental Protection Agency
FAME	Fatty acid methyl esters
FC	Field capacity
FID	Flame ionization detector
G	Guanine
GC	Gas chromatography
IGS	Intergenic spacer
MPN	Most probable number
MTY	Modified tryptone yeast extract agar
N	Nitrogen
NCBI	National Center for Biotechnology Information
NMT	N-metyltriazolinone
NODT	N,O-dimethyltriazolinone
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PLFA	Phospholipids fatty acids
ppb	Parts per billion
PVPP	Polyvinylpolypyrrollidone
rDNAs	Ribosomal DNAs
RFLP	Restriction fragment length polymorphism
rpm	Revolutions per minute

SDS	Sodium dodecyl sulphate
SE	Standard error
SOM	Soil organic matter
TSA	Tryptic soy agar
USDA	United States Department of Agriculture
UV	Ultraviolet
$\delta^{15}N$	^{15}N natural abundance

1 INTRODUCTION

Adequate weed control is vital in sustaining high crop yields, and consequently herbicides have become a key component in modern agricultural production. Ideally, herbicides control weeds during the growing season of the treated crop and dissipate to non-toxic levels before the next crop is seeded; however, some herbicides may persist longer than desired and injure or kill subsequently planted crops (Hanson et al., 2004). Such persistence of residual herbicides in the soil is a disadvantage for producers who aim to maximize crop diversity and productivity through rotation because it limits the flexibility of crop rotation planning (Beckie and McKercher, 1989). Furthermore, when herbicides are applied, most of the spray solution contacts the soil and may affect soil microorganisms that are important for sustainable agriculture, e.g., recycling of plant nutrients, maintenance of soil structure, and symbiotic assistance of crop growth (Vieira et al., 2007). The impact on soil microbes can occur instantly at the time of application and/or have long-lasting effects into the following cropping years.

In the spring of 2000, the active ingredient flucarbazone-sodium and the associated end-use product Everest® were registered for use in Canada as a selective post-emergence herbicide for the control of wild oats (*Avena fatua* L.) and green foxtail (*Setaria viridis* L.) in spring wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.) (Pest Management Regulatory Agency, 2000). Upon registration, the residual properties of the herbicide were extensively analyzed from the stand point of environmental protection and food/feed safety by the federal regulatory body (Pest Management Regulatory Agency, 2000). However, the full impact of the residual

properties of this herbicide on agricultural production is still under investigation. For instance, there is a lack of published data on the potential influence of residual flucarbazone on the success of *Rhizobium* inoculation on grain legume production.

In western Canada the chief cereal crop, wheat, is often followed with annual grain legumes such as field pea (*Pisum sativum* L.), chickpea (*Cicer arietium* L.), and lentil (*Lens esculenta* L.) in a sequential rotation. This type of crop rotation is beneficial in conservation and replenishment of soil nitrogen (N) based on the leguminous plants ability to fix atmospheric nitrogen (N_2) through symbiotic association with effective strains of *Rhizobium* bacteria (Baldock et al., 1981; Wright, 1990; Beckie and Brandt, 1997; Przednowek et al., 2004). As a result, production of grain legumes has increased in western Canada over the years. In particular, the cropping area for field pea in the prairie provinces increased from 25 000 ha in 1976 to almost 1.5 million ha in 2007 (Clayton et al., 2004b; Statistics Canada, 2007), which makes field pea one of the most widely grown legumes in the area.

During field pea production, farmers in the prairie region commonly use some form of commercial *Rhizobium* inoculant. There may be native strains of rhizobia naturally present in soil and these organisms may be able to establish a symbiotic association with field pea. However, native strains may not necessarily be efficient N_2 fixers. Thus, the purpose of inoculation is to establish highly effective inoculum strains in the rhizosphere so that they can compete successfully for infection sites on root surfaces against native strains (Dowdle and Bohlool, 1987). Rhizobia can commence N_2 fixation only after successful nodule formation. As a result, inoculation will not be effective unless the inoculum rhizobia dominate a significant portion of nodule sites on host plant roots, and subsequently initiate root nodule formation. Because the symbiotic association between the host legume and the *Rhizobium* is sensitive to changes in many

environmental factors and soil conditions, it follows that agrochemical residues in the soil may influence inoculation success, N_2 fixation, and yield of field pea.

Flucarbazone belongs to a group of herbicides that contains an active ingredient that interferes with an important metabolic pathway (i.e., branched-chain amino acid synthesis) unique to plants and microorganisms (Royuela et al., 1998). Thus, the product label provides specific instructions on how to minimize the chance of crop damage from residual flucarbazone in the sequential season, yet no information is provided on its potential influence on soil microbial activities and symbiosis. Consequently, the potential toxicity to *Rhizobium* bacteria, as well as the risk of negatively impacting plant-microbe interactions, has to be considered when using these herbicides (Zawoznik and Tomaro, 2005). Furthermore, given the residual nature of flucarbazone and its increasing usage in crop rotation preceding field pea, there is a need to understand if and how the herbicide residue may influence the inoculation success in field pea production.

In order to elucidate the effect of residual flucarbazone on the inoculation success in field pea production, this study tested the following null hypothesis: *The presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia*. The two main goals of the research were to examine the effect of flucarbazone application on the various parameters of successful field pea production, and to investigate the residual behaviour of flucarbazone and its influence on the native rhizobial population. These goals were accomplished through a series of field plot and growth chamber studies designed to:

1. Determine the effect of flucarbazone application on growth parameters, N_2 fixation, and nodule occupancy in field pea;
2. Measure residual flucarbazone carryover in previously treated soils; and
3. Evaluate the effect of flucarbazone application on the native rhizobial population.

2 LITERATURE REVIEW

2.1 Flucarbazone-sodium: An AHAS-inhibiting Herbicide

Flucarbazone-sodium belongs to the chemical family sulfonylaminocarbonyl-triazolinone. Like other Group 2 herbicides, such as imazethapyr (Pursuit®) and sulfosulfuron (Sundance®), flucarbazone exerts phytotoxic effects on sensitive plants by blocking the normal functioning of an enzyme called acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS) (Weed Science Society of America, 2002). This enzyme, which is found in bacteria, fungi, algae, and plants, catalyzes the first step in the synthesis of branched-chain amino acids valine, leucine, and isoleucine (Duggleby and Pang, 2000). The precise mechanisms that link AHAS inhibition with plant death have not been elucidated, yet Gaston et al. (2002) suggested a mechanism where the impairment of AHAS activity leads to a fermentative metabolism in the plant which, in turn, causes growth inhibition and plant death.

AHAS-inhibiting herbicides are generally used at very low concentrations given their acute toxicity to plants (Santel et al., 1999). Absorption occurs through the foliage and the root system, followed by translocation within the plant (Santel et al., 1999). The inhibition of the amino acid synthesis initially occurs at the rapidly growing regions of the plant, spreads to other mature tissues, and ultimately leads to the termination of plant growth (Kishore and Shah, 1988). Given their persistence in soil, these herbicides not only control weeds that already have emerged, but also provide control over weeds that emerge after the time of application through root uptake (Vencill, 2002). Though such extended weed control is desirable, when environmental and soil conditions are such that

the herbicide does not dissipate to a non-toxic level before the next crop is seeded, it can cause injury to rotational crops (Moyer, 1995; Moyer and Esau, 1996). It has been reported that extremely low levels of AHAS-inhibiting herbicides, when received by a susceptible species during reproductive growth, could have devastating effects on seed production without causing pronounced visual symptoms (Bhatti et al., 1995; Fletcher et al., 1996). The residual nature of flucarbazone and its potential negative impact on pea production is further investigated in the following sections.

2.2 Factors Affecting the Occurrence of Crop Injury Caused by Residual Herbicide

The potential for crop injury from residual herbicide is determined by the persistence of a given herbicide in soil, the bioavailability of the herbicide, and the susceptibility of a crop to the herbicide (Hartzler et al., 1989).

2.2.1 Persistence of flucarbazone in soil

AHAS inhibitors can persist in soil from a couple of days to years (Colborn and Short, 1999), and the rate at which the herbicide dissipates to a non-toxic level is significantly influenced by environmental and soil conditions (Eliason, 2003). More specifically, environmental conditions influence degradation and translocation which are the two common ways in which applied herbicides dissipate from the soil.

Degradation alters the chemical structure and properties of a herbicide, and new (generally less toxic) compounds are formed. This may occur by exposure to sunlight, chemical reaction, or microbial activities (Hanson et al., 2004). While all herbicides potentially are susceptible to decomposition upon exposure to sunlight (i.e., photodecomposition), the effect is much greater for some herbicides than others (Griffin, 2006). With flucarbazone, photodecomposition in soil reportedly is not significant (EPA, 2000). Degradation of herbicides through chemical reaction commonly takes place when

herbicides react with soil water and hydrolysis occurs (Hanson et al., 2004); therefore, dry soil conditions generally prolongs herbicide persistence (Eliason, 2003).

With flucarbazone, the primary route of degradation is believed to be microbial (Santel et al., 1999) where bacteria and fungi in the soil use various enzymes to degrade herbicides and other compounds to obtain nutrients (Hanson et al., 2004). Generally, environmental factors such as warm soil temperature, adequate soil moisture, and high soil organic matter encourage microbial growth and reproduction (Griffin, 2006), which in turn provides an ideal environment for microbial degradation of herbicides. In contrast, environmental conditions that are not favourable for microbial proliferation reduce the rate of degradation.

The product label for Everest® states that the herbicide is degraded by soil microbes (Arysta LifeScience, 2007). It cautions farmers making rotational cropping decisions to be mindful of environmental conditions that may decrease microbial activities in soil. These conditions include prolonged drought and/or cold temperatures within the following cropping season, as well as soils with both low organic matter (less than 2%) and high pH (greater than 7.5). Because such conditions can result in greater amounts of residual flucarbazone carried over into the subsequent growing season, the herbicide manufacturer encourages farmers in the described situation to have the field tested using a bioassay to ensure safety for the rotational crop.

Another pathway of herbicide dissipation is translocation of the herbicide from the site of application, such that it becomes inaccessible to plants and microbes. This process includes volatilization, surface erosion, removal from the site by plant/animal uptake, leaching through soil, and adsorption to soil (Hanson et al., 2004), where adsorption can be considered translocation at a microscopic level. While surface erosion and plant/animal uptake are highly unpredictable, volatilization losses of flucarbazone are consistently negligible because it is a non-volatile compound (EPA, 2000). As for

leaching, Whitcomb (1999) reviewed characteristics of various AHAS inhibitors and reported the long-term stability and high mobility of some of the AHAS-inhibiting herbicides in soil. Whitcomb (1999) reported that in areas where water tables are shallow and soils are near neutral or higher in pH, these herbicides move deeper with rainfall but move back near the soil surface with rising water table or soil drying via capillarity, and they can remain in the soil solution indefinitely.

2.2.2 Bioavailability of flucarbazone in soil

Sorption–desorption interactions of herbicides with soil determine the availability of the chemical in soil (Koskinen et al., 2006). It has been reported that the bioavailability of herbicide in soil is strongly influenced by the soil organic matter (SOM) content because soils with high SOM content have increased herbicide retention capacity due to the presence of a large number of adsorption sites (Loux et al., 1989). Also, it has been observed that SOM adsorbed herbicide is unavailable for plant uptake and thereby unable to cause plant injury (El-Azzouzi et al., 1998). Based on the root length inhibition results from a mustard bioassay, Eliason (2003) reported the presence of inverse relationship between SOM content and flucarbazone phytotoxicity in nine soils from western Canada.

Generally considered to have less influence on chemical adsorption than SOM, soil texture (i.e., relative composition of sand, silt, and clay) also has been known to play an important role in herbicide adsorption (Griffin, 2006). In fact, herbicide application rates sometimes vary according to soil type, and are generally higher for soils with high clay content compared with low clay soils because clay provides much greater soil surface area than sand for chemical adsorption (Calvert, 1980; Peter and Weber, 1985). In the case of flucarbazone, Eliason (2003) reported for nine western Canadian soils that

the effect of soil texture appeared to be overshadowed by SOM content in its influence on adsorption and phytotoxicity of flucarbazone in soils.

2.2.3 Susceptibility of pea to flucarbazone

Plant species vary in their sensitivity to AHAS inhibitors. For example, the amount of an AHAS-inhibiting herbicide (chlorsulfuron) needed to cause 50% reduction in the growth of a tolerant and a non-tolerant weed species was found to be different by 21,000-fold (Hageman and Behrens, 1984). Such a large difference was explained by the fact that the chlorsulfuron-tolerant plants (such as wheat, oats, and barley) rapidly metabolize the herbicide to an inactive product, while sensitive broadleaf plants show little to no metabolism of chlorsulfuron (Sweetser et al., 1982).

The product label for Everest® lists recommended rotational crops to be grown in the year following flucarbazone application (Arysta LifeScience, 2007). For example, the recommendation for the Black soil zone includes: spring wheat, durum wheat, barley, canola, field bean and flax. Field peas are also recommended for Dark Brown, Black, and Gray-Wooded soil zones, but with additional safety precautions. For example, crop safely is favoured only if precipitation has been normal or above normal (10 year average) during the growing season, pH is below 7.5, and organic matter content is above 4% (Arysta LifeScience, 2007).

In an unpublished field experiment, Sapsford et al. (2006) compared the sensitivity of fifteen crops to residual flucarbazone in field plots, eleven months after the initial application of the herbicide at various rates (0, 15, 20, 30, 40, and 60 g a.i. ha^{-1}) in wheat, where 30 g a.i. ha^{-1} is the recommended field application rate. They observed no visual injury in wheat, durum wheat, flax, and canola in the year following application even when these crops were grown on soil that received double the recommended rates (60 g a.i. ha^{-1}) of flucarbazone in year 1. On the other hand, yellow mustard, juncea

mustard, chickpea, and corn, grown on plots that received half the recommended concentration (15 g a.i. ha^{-1}) of flucarbazone in year 1, showed visually notable injury. The sensitivity of field pea fell somewhere in between these two groups, where no injury was observed when grown on plots that received 40 g a.i. ha^{-1}; however, visually notable injury was present on plots that received 60 g a.i. ha^{-1} flucarbazone in year 1. Because precipitation during the growing season greatly affects the extent of injury, year-to-year variation is expected on the sensitivity of these crops to the herbicide; however, the data provides a valuable perspective into the relative sensitivity of crops commonly grown in the region to residual flucarbazone.

2.2.4 Challenges in studying flucarbazone residues and its degradation products

Some AHAS inhibitors not only persist in soil for a long period of time, but the residual levels are such that in most cases, it cannot be detected analytically (Whitcomb, 1999). According to Whitcomb's review (1999) it has been found that traditional chemical analytical methods were of limited value because they could not detect concentrations of AHAS inhibitors still herbicidally active in the soil. For instance, Wheeler and McNally (1987) tested three analytical procedures for detecting AHAS inhibitors (sulfonylureas) in soil, and found that the lowest detection level was 1 μg g^{-1}, which is 40 000 times higher than the concentrations reported by Brewster and Appleby (1983) for one of the sulfonylureas to cause crop injury. For this reason, the use of bioassays has been proposed to monitor soil residues in order to predict the risk of injury to rotational crops.

To further complicate the matter, the degradation of flucarbazone leads to the formation of several chemical compounds (i.e., metabolites) such as sulfonamide, sulfonic acid, and O-desmethyl flucarbazone (EPA, 2000). While sulfonamide and sulfonic acid are resistant to aerobic metabolism in soil, O-desmethyl flucarbazone is

rapidly hydrolyzed to N,O-dimethyltriazolinone (NODT) which itself degrades rapidly to N-metyltriazolinone (NMT). The latter is either immobilized in the soil or metabolized to CO_2 (EPA, 2000).

Given the lack of published data on these metabolites, it is difficult to predict their influence on plants and soil microorganisms, and the possibility exists that these metabolites could interfere with biological processes. Of the three flucarbazone metabolites reported by the EPA, sulfonamide would likely have the greatest potential to negatively influence the inoculation success during field pea production as it can persist in the field for over one year (EPA, 2000). Furthermore, various sulfonamide compounds have been used as synthetic antimicrobial agents (i.e., sulfa drugs) in inhibiting the growth of a large number of Gram-positive and Gram-negative bacteria (Accinelli et al., 2007). Anccinelli (2007) studied the influence of sulfonamides (sulfamethazine and sulfachloropyridine) on the soil microbial community and reported that concentrations up to 0.1 g kg^{-1} had no effect on the soil microbial community.

2.3 Influence of Flucarbazone on the Legume-rhizobia Symbiosis

Herbicides may affect the legume-rhizobia symbiosis in a number of ways including: (i) direct effects on the host plant (e.g., reduction in root biomass, leading to fewer infection sites, or reduced carbohydrate supply to existing nodules); (ii) direct effects on rhizobial survival or growth, leading to a decreased potential for rhizobial infection of root hairs; (iii) an inhibition or inactivation of the biochemical signalling by either rhizobia or plants required to initiate nodule development; and/or (iv) an inhibition of nodule development by reducing the capacity for cell division (Eberback, 1993). Though published data specifically dealing with the influence of flucarbazone on legume-rhizobia symbiosis are not available at the time of writing, the impact of other AHAS-inhibiting herbicides on N_2-fixing symbiosis has been studied.

The growth of soybean rhizobia (*Bradyrhizobium japonicum*) was not affected when 150 times the recommended field application rates of chlorimuron-ethyl was added to the pure culture; however, the number of nodules formed on soybean plants was significantly reduced in a pot experiment when the plants were treated with the herbicide at standard application rates five days after emergence (Zawoznik and Tomaro, 2005). Interestingly, the herbicide-treated soybean plants formed fewer but more active nodules, and there was no difference in nodule size or the shoot N content between herbicide-treated and control plants. These findings led Zawoznik and Tomaro (2005) to speculate that the influence of chlorimuron-ethyl on root tissue or on a bacterial partner at early stages of the nodule formation process caused abortion of developing nodules. Gonzalez et al. (1996) similarly reported that the number of nodules present on pea roots was reduced by imazethapyr whereas nodule size was unaffected, suggesting a direct imazethapyr effect on the initiation of nodulation rather than on later developmental stages. Anderson et al. (2004) reported that the addition of chlorsulfuron at double the recommended field application rate did not influence chickpea rhizobia (*Mesorhizobium ciceri*) grown in medium containing no external source of amino acids. However, the number of nodules formed was reduced when germinating chickpea seeds were inoculated with rhizobia that were briefly pre-exposed to chlorsulfuron. This led Anderson et al. (2004) to speculate the possibility of herbicidal interference with the nodule infection and development processes.

2.4 Influence of Flucarbazone on Competition and Occupation of Nodules by Inoculum Strains

The reduction in nodule count on host plant roots upon herbicide treatment, as observed by Anderson et al. (2004), Gonzalez et al. (1996), and Zawoznic and Tomaro (2005), was based on gnotobiotic studies where growth media, nutrient solution, and

seed surface were sterilized prior to addition of pure *Rhizobium* culture so that the only bacterium present in the system was the added inoculum strain. When producers apply commercial inoculant during field pea production, there are abundant soil bacteria present in soil which may contain native strains of rhizobia capable of forming a symbiotic relationship with field pea. In western Canada, the presence of native field pea rhizobia (*R. leguminosarum bv. viceae*) in soil is highly probable (Rennie and Dubetz, 1986; Bremer et al., 1988; Kucey and Hynes, 1989; Clayton et al., 2004b). This makes the assessment of residual herbicidal influence on inoculation success much more complicated.

Upon introduction of inoculum rhizobia into a multi-strain field environment, they firstly have to survive and adapt to the prevailing environmental conditions, then multiply and function as part of the overall soil microbial population until the roots of the host plant emerge (Pan and Smith, 2000). Having successfully survived independent of the host plant, the inoculant rhizobia then need to compete on the roots for infection sites against indigenous rhizobia (Streit et al., 1995). If the inoculum strain is inferior to native rhizobia in these steps (e.g., due to differential sensitivity of rhizobia to the herbicide), they will have less chance to occupy a significant proportion of the nodules, and a reduced contribution to N_2-fixation can be expected from inoculation.

Forlani et al. (1995) studied the differential sensitivity of plant-associated bacteria to AHAS-inhibiting herbicides and reported that the presence of rimsulfuron significantly promoted root colonization by the resistant bacterial strain when maize seedlings were inoculated with two strains, one tolerant and one sensitive to the herbicide. They concluded that the AHAS-inhibiting herbicides tested (chlorsulfuron, rimsulfuron, imazapyr, and imazethapyr) can influence the microbial structure of the rhizosphere at recommended rates of application (Forlani et al., 1995).

2.5 Methods for Analyzing Nodule Occupancy

When several rhizobial strains occur in the presence of the leguminous plant to which they are specific, certain strains form nodules in preference to others (Amarger and Lobreau, 1982). This is because rhizobial strains differ from one another in their ability to be selected by the plant host (Amarger and Lobreau, 1982). Since rhizobia can commence N_2 fixation only after successful nodule formation, inoculation will not be effective unless the inoculum rhizobial strain dominate nodule sites on the host plant roots, then form and occupy a significant proportion of the total nodules against native strains. To monitor inoculation success of agriculturally important leguminous crops, various strategies have been developed to determine the identity of the nodule occupants. These techniques can be broadly classified into biochemically-based or nucleic acid-based methods.

2.5.1 Biochemically-based methods

Commonly used biochemical identification methods of nodule occupants are based on either the antibody/antigen reaction (e.g., immunofluorescence, Enzyme-Linked Immuno Sorbent Assay which is often abbreviated as ELISA) or the analysis of microbial membrane lipids, namely phospholipids fatty acids (PLFA) or fatty acid methyl esters (FAME). Antibody based methods, which exploit the specificity of antibodies against surface constituents of a cell, have been successfully used for the detection of nodule occupants in various legumes (May and Bohlool, 1983; Trinick et al., 1989; Revellin et al., 1998). However, it requires the preparation of specific antibodies against the target organism, which is a time consuming and laborious procedure (Madigan et al., 2003b). Also, the potential cross reactions of antibodies with non-target bacterial strains reduce its specificity (Stead et al., 2000).

Fatty acid profiling utilises the variability in the composition and proportion of bacterial membrane lipids. Since such lipid biomarkers differ among microbial genera and species, it is possible to construct a reference database to which individual sample isolates can be compared for taxonomic identification (Spiegelman et al., 2005). Generally, fatty acid profiling is a very cost effective method of identification down to species level because species within a genus often contain the same types of fatty acid but in different proportions (Stead et al., 2000). However, overlap of fatty acid characteristics can occur at subspecies level among microorganisms of genetic variant or subtype (i.e., strains), and accuracy of identification may decrease (Stead et al., 2000).

2.5.2 Nucleic acid-based method

In an attempt to differentiate nodules occupied by inoculum rhizobia from those colonized by native rhizobia, a method that has the potential to discriminate a strain level variation is desirable. Since the differences in strains arise from the genetic variation, nucleic acid-based methods that directly analyse the genetic materials are suitable for such an endeavour. There are numerous ways in which bacterial genetic materials can be used in microbial identification. The procedures fundamental to most of the commonly used methods are: 1) exposure/extraction of the whole bacterial genetic material (i.e., genome) through physical or chemical disturbance of cell membrane; 2) use of polymerase chain reaction (PCR) to obtain multiple copies of a certain section of the genome; and 3) analysis of the amplified segment and its similarity/difference to the equivalent sections amplified from other bacterial samples of interest.

2.5.2.1 Choosing the target genetic region to be amplified

The similarities/differences among various bacteria is dependent on their evolutionary relatedness because evolution involves random alteration in gene sequences, and it causes change in a line of descent leading to the production of new species or strains within species (Madigan et al., 2003a). In studying such phylogenetic relationships, the gene section analyzed should display molecular clock-like behaviour where the rate of change coincides with evolution, and have large enough size to provide adequate amounts of information on gene sequence variation (Woese, 1987). It is also important that the gene section is universally distributed across the group chosen for study, and it is moderately well conserved so that the evolutionary relatedness among various bacteria can be extrapolated from the degrees of similarities and differences of the gene sequences within the amplified sections (Madigan et al., 2003a).

Given their crucial role in protein-synthesis, the genes that encode ribosomal RNA (i.e., ribosomal DNAs or rDNAs) are universally distributed and moderately well conserved in sequence across broad phylogenetic distances (Madigan et al., 2003a). All three rDNAs (5S, 16S, and 23S) collectively comprise the ribosomal RNA operon (i.e., the cluster of functionally related genes regulated and transcribed as a unit), which instruct the production of 5S, 16S, and 23S ribosomal RNAs that jointly make up a molecule of ribosome.

The ribosomal operon is a classic molecular marker used to trace genetic relationships. Particularly, the 16S rDNA has been used extensively to characterize soil microorganisms, including rhizobia (Marilley et al., 1998; Garbeva et al., 2001; Young et al., 2004; Corgie et al., 2006). Though 16S rDNA contains several regions of highly conserved sequences that mutate very slowly, it also contains relatively variable regions which can allow discrimination at the genus and species levels (Giovannoni et al., 1988; Rome et al., 1997; von Wintzingerode et al., 1997).

When 16S rDNA does not provide sufficient resolving power to distinguish closely related organisms, the use of alternative gene sections has been proposed. Of all the different regions of the ribosomal operon, the intergenic spacer (IGS) regions between 16S and 23S ribosomal DNA are frequently used as molecular markers to identify bacteria, and analyze the phylogenetic relationship between strains (Gürtler and Stanisich, 1996; Daffonchio et al., 1998). The usefulness of the intergenic spacers primarily stems from its variability in sequence and length. Due to a higher mutation rate, intergenic spacers are relatively more variable in gene sequence than adjacent genes (Daffonchio et al., 1998). Also, it has been reported that the length of spacer region varies among bacteria from 50 base-pair (bp) to 1500 bp (García-Martínez et al., 1999; Ranjard et al., 2001). Such two-fold variability allows for more detailed taxonomic identification than can be attained by the use of more conserved regions.

2.5.2.2 Analyzing the amplified target genetic region

Once multiple copies of the target region are obtained through PCR amplification, the next step is the comparison and differentiation of target regions obtained from various sample bacteria. If one chooses not to have the samples sequenced at this point, the potential gene sequence variation among samples needs to be visualized and expressed in the form of various migration/banding patterns on a gel (i.e., electrophoresis). Unfortunately, 16S rDNA samples cannot be differentiated based on their size without additional manipulation because 16S rDNA is extremely constant in size across a wide range of bacteria (García-Martínez et al., 1999). Thus, 16S rDNA samples need to be processed further by using such techniques as: 1) restriction fragment length polymorphism (RFLP) which involves pre-electrophoresis digestion of amplified rDNA samples with a set of enzymes that cut/restrict rDNA into a set of shorter strands

of unique lengths, depending on where the restriction enzymes cut a given rDNA strand; or 2) denaturing gradient gel electrophoresis (DGGE) which is a special gel electrophoresis method that can differentiate rDNA strands of comparable length in terms of their base sequence variation (i.e., G-C content).

In contrast, the analysis of PCR-amplified ribosomal intergenic spacer does not require restriction digestion or denaturing gradient gel electrophoresis (Spiegelman et al., 2005). Owing to its sequence and length variability, as well as the variation in the number of ribosomal RNA operon present in various bacteria (Gürtler, 1999), the amplified intergenic spacer products produce unique banding patterns when separated by agarose gel electrophoresis (Cartwright et al., 1995; Khbaya et al., 1998; Flint et al., 2001). Although it is possible to use restriction enzymes or denaturing polyacrylamide gel electrophoresis on amplified intergenic spacer products to achieve greater resolution, these procedures add extra incubation time, multiple digestions, and preparation of special gels with long gel-running time, which are not suitable when a large number of samples need to be processed and analyzed. In contrast, agarose gel electrophoresis is relatively simple to conduct and is thus more suitable for repeated analysis. Also, Khbaya et al. (1998) analyzed genetic diversity and phylogeny of rhizobia and reported that the digestion of amplified 16S-23S spacer samples with nine restriction enzymes did not allow them to make a clearer distinction among the strains.

The above review of the various methods used in analyzing nodule occupancy highlights the pros and cons of those procedures commonly employed. Given the wide range of techniques available to characterize various microorganisms, the potential exists to utilize these methods to study the impact of various environmental factors, including the presence of residual herbicides, on nodule occupancy and *Rhizobium* inoculation success.

3 THE EFFECT OF FLUCARBAZONE APPLICATION ON GROWTH PARAMETERS, NITROGEN FIXATION, AND NODULE OCCUPANCY OF FIELD PEA: GROWTH CHAMBER EXPERIMENT

3.1 Introduction

In order to minimize rotational crop injury and maximize economic yield from crop rotations, it is important to consider soil persistence characteristics of herbicides because some herbicides can potentially carry over and injure subsequently planted crops. In western Canada where wheat production is often followed with annual grain legumes such as field pea (*Pisum sativum* L.), chickpea (*Cicer arietium* L.), and lentil (*Lens esculenta* L.) in a sequential rotation, it is particularly important for the producers to be aware of the residual properties of herbicides. This is because the symbiotic association between the host legume and the *Rhizobium* bacteria is sensitive to changes in many environmental factors and soil conditions, and thus agrochemical residues in the soil may influence inoculation success, N_2 fixation, and yield of grain legumes.

Amongst many herbicides used in wheat, acetohydroxyacid synthase (AHAS)-inhibiting herbicides warrant special attention given their residual properties (Colborn and Short, 1999), acute plant toxicity at low concentrations in soil (Santel et al., 1999), and their potential ability to influence both host plant and the bacterial symbiotic partner (Duggleby and Pang, 2000). In fact, the reduction in plant/nodule production and N_2 fixation induced by various AHAS-inhibiting herbicides in different leguminous plants has been reported (Gonzalez et al., 1996; Royuela et al., 2000; Anderson et al., 2004; Zawonznik and Tomaro, 2005).

Flucarbazone-sodium is a relatively new AHAS-inhibiting herbicide used in western Canada to control wild oats (*Avena fatua* L.) and green foxtail (*Setaria viridis* L.) in spring wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.) (Pest Management Regulatory Agency, 2000). Despite the herbicide's popularity in the region, published data specific to the influence of flucarbazone on grain legume production, nodule production, and N_2 fixation is not available at the time of writing. Thus, a growth chamber study was conducted to elucidate the influence of flucarbazone on the shoot and root development, nodule production, N_2 fixation, and inoculation success during production of field pea.

This experiment was conducted under controlled plant growth conditions and used a wide range of herbicide application rates in an attempt to determine the concentration at which residual levels of the herbicide significantly influences the above mentioned parameters of field pea production. It was recognized that flucarbazone freshly added to the soil for this experiment may not simulate the residual flucarbazone in the field because it has been reported that the aging of flucarbazone in the field leads to the formation of several chemical compounds (i.e., metabolites) such as sulfonamide, sulfonic acid, and O-desmethyl flucarbazone (EPA, 2000). However, this experiment was important in establishing the flucarbazone concentration at which field pea production is negatively influenced, and also to gain a general appreciation of plant and rhizobial response to the presence of an AHAS-inhibiting herbicide.

3.2 Materials and Methods

3.2.1 Experimental setup

Soil samples were collected from experimental field sites established at the University of Saskatchewan Goodale Crop Research Farm, Saskatchewan (SW3-36-4-W/3). The site was established as a component of the field experiment portion of this

thesis project, and soils used for the growth chamber experiment were collected from within the control plots of the field experiment. Detailed descriptions of the field site are provided in Chapter 4. Briefly, the field site was established and seeded to wheat in 2005 (year 1). To manage weeds, the control plots were treated with a non-residual herbicide containing no AHAS inhibitor, while the rest of the plots were sprayed with various rates of flucarbazone-sodium to simulate flucarbazone carryover into the subsequent field pea cropping season in 2006 (year 2). The soil samples for the growth chamber experiment were collected from the control plots in year 2, prior to seeding to pea.

Soil samples were collected from each of the four replicated control plots with a shovel to the depth of 10 cm, put into a container, and thoroughly mixed to obtain a composite sample. The sample was then air dried in the laboratory, sieved to pass through a 2-mm mesh, and then dispensed into 15-cm diameter plastic pots at 1.2 kg soil per pot. Each pot was lined with a plastic bag to prevent nutrient solution leaching. Field capacity (FC) moisture content of the soil was approximated according to Eliason et al. (2004) by determining the volume of water required to completely wet the known volume of air-dried soil to the bottom of a 100 mL plastic vial.

In order to apply nutrients and flucarbazone to the pots, micro/macro nutrient solutions and various concentrations of flucarbazone solutions were prepared (Appendix A). Appropriate amounts of each solution (Appendix A) were mixed with water in a beaker, and applied to each pot to attain 75% FC and final flucarbazone concentrations ranging from 0 to 40 μg kg^{-1} in 5 μg kg^{-1} increments. The highest application rate (40 μg kg^{-1}) is equivalent to twice the recommended field application rate of 30 g active ingredient per hectare (a.i. ha^{-1}), which approximately converts to 20 μg kg^{-1}. The spiking concentration range was chosen to ensure that sufficient herbicide would be present in at least some of the treatments to cause a negative impact on plant growth, nodulation, and nodule occupancy, particularly at the higher application rates. Each

herbicide application rate was replicated four times, and a total of 36 pots were set up (i.e., 9 herbicide rates x 4 replicates = 36 pots). Moistened soil was thoroughly mixed in the plastic bag used to line the pot to ensure even distribution of the nutrients and herbicide throughout the pot. Each pot was then seeded with four, pre-germinated field pea (*Pisum sativum* cv CDC Mozart) seeds at a depth of 2.5 cm.

Prior to pre-germination, the seeds were surface-sterilized by soaking in 95% ethanol for 10 s, followed by a 3 min soak in full-strength, household bleach (i.e., 5% sodium hypochlorite; NaOCl). The seeds were then rinsed with sterile dH_2O six times where fresh sterile water was used each time. The water used in last rinsing (0.1 ml) was plated on 1/10 tryptic soy agar (TSA) plate to ensure the seed surface sterility, then the seeds were allowed to germinate in a Petri dish with 10 ml of fresh sterile dH_2O, lined with a sterile filter paper. Five seeds were placed per Petri dish.

Seeds were inoculated with a strain of *R. leguminosarum* bv. *viceae* pure culture, grown in yeast mannitol broth (i.e., 1/10 YMB), at a rate of approximately 4.3×10^8 colony forming units (cfu) mL^{-1} $seed^{-1}$, by pipetting 1 mL of culture directly onto the seed in the soil. The seeds were then covered with soil.

The *Rhizobium* used in this experiment is one of the two *R. leguminosarum* bv. *viceae* strains present in a commercial peat inoculant (NitraStik-C®; EMD Crop BioScience, WI). The peat inoculant was also used in the field experiment (Chapter 4), and the inoculum rhizobia are referred to as reference strains R_1 and R_2 where R_1 is the strain used in this growth chamber experiment. The pure cultures of each strain were generously provided by EMD Crop BioScience.

Following the emergence of the cotyledon, each pot was thinned to two plants, tomato cages were added for support, and the soil surface was covered with a 0.5-cm layer of white plastic beads to minimize soil drying. Pots were watered regularly to maintain 75% FC by weight during early stages of plant growth. When plant

transpiration became significant, they were watered to 100% FC on a daily basis. Pots were kept in a growth chamber with a 16 h photoperiod and day/night temperatures of 22/18 ºC. Weed seedlings were removed daily and pots were regularly rotated under the light canopy which provided photon flux density of approximately 300 µmol m^{-2} s^{-1}.

3.2.2 Observation of shoot/root biomass, and nodule formation

While at the flowering to pod formation stage, plants were harvested. The roots were washed with a gentle stream of tap water, and the whole intact plant was air-dried at room temperature. Once a constant weight was achieved, shoots were separated from roots, and nodules were removed from roots. Above-ground biomass (i.e., shoots and pods), root biomass, nodule number, and nodule weight were recorded for every two plants harvested from each pot.

3.2.3 Assessment of nitrogen fixation by ^{15}N natural abundance method

Above-ground biomass samples (i.e., shoots and pods) were roughly ground in a grinder, and re-ground in a rotating ball-bearing mill into fine powder. Subsamples (1 ± 0.05 mg) were analysed for natural abundances (δ) of the stable isotope pair $^{15}N/^{14}N$ using a ANCA elemental analyzer coupled to a TracerMass 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 (REF/13CN.PEAGRN) with an atom % ^{15}N of 0.3675 was used as a working standard. The $\delta^{15}N$ value was calculated according to Robinson (2001) as:

$$\delta^{15}\mathrm{N} = 1000\left(\frac{\mathrm{R}_{\mathrm{sample}} - \mathrm{R}_{\mathrm{standard}}}{\mathrm{R}_{\mathrm{standard}}}\right) \qquad (3.1)$$

where $R_{standard}$ stands for the $^{15}N:^{14}N$ isotope ratio of atmospheric N_2 ($R_{standard}$ = 0.0036637) with $\delta^{15}N$ of 0 ‰, and R_{sample} is the isotope ratio of the sample.

3.2.4 Nodule occupancy analysis

To conduct nodule occupancy analysis, a simple and rapid method was required to identify the origin of rhizobial nodule occupants as either inoculum or non-inoculum rhizobial strains. Prior to the adaptation of the molecular microbiological method as described in Chapter 3 and 4, the use of FAME (fatty acid methyl ester) profiling was considered. However, after a preliminary experiment (Appendix C), it became evident that the FAME profiles of the rhizobial strains of interest were too similar to be identified as distinct strains. Since strain level identification was necessary for the present study, the use of FAME analysis was not explored further, and molecular biological method was adopted instead.

3.2.4.1 DNA extraction

Following the removal of nodules from the roots, nodule samples were grouped into crown and distal regions to accommodate separate analysis of nodule occupants in these regions. Crown region was defined as roots within a 3-cm radius around the seed. Nodules on the roots outside of this radius were grouped as distal nodules.

The method described by Santasup et al. (2000) was the original protocol used for DNA extraction from dried nodules, but in our hands, recovery was inconsistent. The final, optimized DNA extraction method included elements from Santasup et al. (2000) and Berthelet et al. (1996). The modifications were as follows.

For every two plants grown in each pot, 12 nodules were selected (i.e., six crown and six distal), soaked in sterile distilled water until they became fully swollen and re-

hydrated. To surface sterilize, re-hydrated nodules were soaked in 95% ethanol for 5 min and briefly dried on a sterile filter paper. Each surface sterilized nodule was placed in a 1.5 mL Eppendorf tube, and crushed mechanically with a sterile plastic rod in the presence of Proteinase K enzyme (5 µL) to promote microbial cell membrane digestion. To physically disrupt the rhizobial cell membrane, sodium phosphate buffer (200 µL), 20% Sodium dodecyl sulphate (SDS; w/v: 20 µL), zirconia/silica beads (0.4 g) were added into each tube, and vortexed for 5 min at maximum speed.

To recover the buffer containing rhizobial DNA clean of the nodule debris, Tris-HCl (200 µL) was added to each tube, centrifuged for 10 min at room temperature (13,000 rpm), and the supernatant transferred to a new 1.5 mL tube. To further precipitate and eliminate protein and cell debris, 7.5 *M* ammonium acetate was added (at half the volume of recovered supernatant), incubated on ice for 15 min, centrifuged for 5 min at 4 ºC (13,000 rpm), and the supernatant transferred into a new 1.5 mL tube. To precipitate DNA out of the buffer for recovery, cold 2-propanol was added to each tube (at the same volume as the recovered supernatant), the tubes were kept overnight at – 20 ºC and centrifuged for 5 min at 4 ºC (14,000 rpm), then the supernatant was discarded, leaving a pellet of DNA at the bottom of each tube. To wash the tube and the DNA pellet, 70% ethanol (500 µL) was added to each tube, gently inverted, centrifuged for 5 min at 4 ºC (14,000 rpm), and ethanol was discarded. After washing the DNA twice in this manner, excess ethanol was allowed to evaporate in a bio-safety cabinet. Finally, the cleaned DNA pellet was dissolved in 50 µL of Tris ETDA buffer (pH 8.0), and passed through a polyvinylpolypyrrolidone (PVPP) spin column to remove chemical substances that can interfere with polymerase chain reaction (PCR) reactions. The PVPP cleaned rhizobial DNA was stored at – 80 ºC until use.

3.2.4.2 PCR amplification

PVPP cleaned DNA isolates were subjected to a PCR reaction with primers FGPS1490 and FGPS132' to amplify the 16S-23S rDNA intergenic spacer (IGS) regions. FGPS1490 is derived from conserved sequences in the 3' part of 16S rDNA genes (Navarro et al., 1992), and reverse primer FGPS132' corresponds to the 5' part of the 23S rDNA gene right next to the IGS (Ponsonnet and Nesme, 1994). To amplify the IGS regions of DNA extracted from nodule samples, 2 µL of PVPP cleaned DNA was 1:100 diluted and used as a template (~ 10 ng μL^{-1}). To be used as a reference during the banding pattern analysis, the reference inoculum strain was also PCR-amplified for the IGS region with the same set of primers by using 2 µL of its bacterial cell suspension as a template.

PCR was done in a 20 µL volume with the aforementioned DNA templates. Taq PCR Master Mix system (Qiagen; Hilden, Germany) was used according to the reaction conditions recommended by the manufacture (1x Qiagen PCR buffer contains 1.5 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase, and 200 µM of each dNTP). The target region was amplified with a Robocycler Gradient 96 (Stratagene; California, USA) using the following conditions: an initial denaturation/cell lysis at 97 ºC for 10 min; 35 cycles of denaturation (1 min at 94 ºC), annealing (1 min at 55 ºC), and extension (2 min at 72 ºC); and final extension at 72 ºC for 3 min.

Amplified PCR products were analyzed by electrophoresis in 2% agarose gels with 10-µL aliquots of PCR products. Gels were stained with ethidium bromide and photographed under UV illumination. By visually comparing the banding pattern of the nodule occupants to the reference inoculum strain, nodule samples were classified as either having 'matching' or 'non-matching' banding patterns as the reference strain.

3.2.4.3 Analysis of nodule occupancy data

For each treatment, four replicated pots were present with each pot containing two plants. From each replicated pot, six nodules were analyzed for each region (crown and distal), and the presence of the inoculum strain was recorded in terms of a fraction as in "X/6" where X signified the number of nodules containing an inoculum strain. To statistically analyze the differences in the proportion of successful colonization by the inoculum rhizobial strain among the treatments, a contingency table was constructed as in the example shown in Appendix B, and a Pearson's chi-square (χ^2) test conducted.

3.2.4.4 Verification of the nodule occupancy analysis

After the completion of visual analysis of agarose gel photographs, a select number of rhizobial DNA extracts from sample nodules were analyzed for their IGS nucleotide sequences, along with that of the reference strain. Sequencing was conducted to verify the accuracy of the visual classification of nodule occupants (i.e., matching or non-matching to the reference strain) on the basis of banding patterns, and to confirm that PCR-IGS products with the same banding patterns are indeed identical in the IGS gene sequences. The verification was jointly conducted for the growth chamber study and the field study. The detailed procedure and the results are provided in Chapter 4. Briefly, select rhizobial DNA extracts from sample nodules and the reference inoculant strains were subjected to PCR reaction as described under Section 3.2.4.2. The PCR-IGS product was cleaned and subsample was taken to be quantified for the DNA concentration. After quantification, the remaining PCR-IGS products were run on 1% agarose gel. A select number of single bands produced by the sample nodules and the reference inoculum strains were excised under the UV illumination, and DNA was eluted from the excised gel and purified. The National Research Council – Plant Biotechnology Institute DNA Sequencing Lab (Saskatoon, Canada) performed the sequencing reactions.

The sequencing results were compared against the microbial nucleotide sequence database to determine the most closely related strain using the National Center for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/).

3.2.5 Statistical analysis

Except for those from molecular analysis of nodule occupancy, data were presented as means and standard errors, and the differences between treatments were assessed using one-way ANOVA with post hoc analysis (Tukey's test) using SPSS (version 15.0.1; SPSS, Chicago, IL). The data from molecular analysis of nodule occupancy were expressed in the form of a contingency table, and Pearson's chi-square (χ^2) test was conducted using SPSS (version 15.0.1; SPSS, Chicago, IL).

3.3 Results

3.3.1 Shoot development and above-ground/root biomass

The influence of flucarbazone addition was evident in shoot development. While the shoot heights of the control plants with no flucarbazone addition steadily increased until harvest (46 days after seeding; DAS) to reach nearly 40 cm on average, the growth of flucarbazone-treated plants was hindered at approximately 25 DAS, and the final shoot heights of all treated plants remained approximately 25% of the control (Fig. 3.1). Furthermore, visual observations at the time of harvest revealed that control plants had healthy leaves and long tendrils, whereas some plants from all flucarbazone-added treatments exhibited browning of the leaf edges and tendril tips which had the appearance of dead plant tissue (Fig. 3.2).

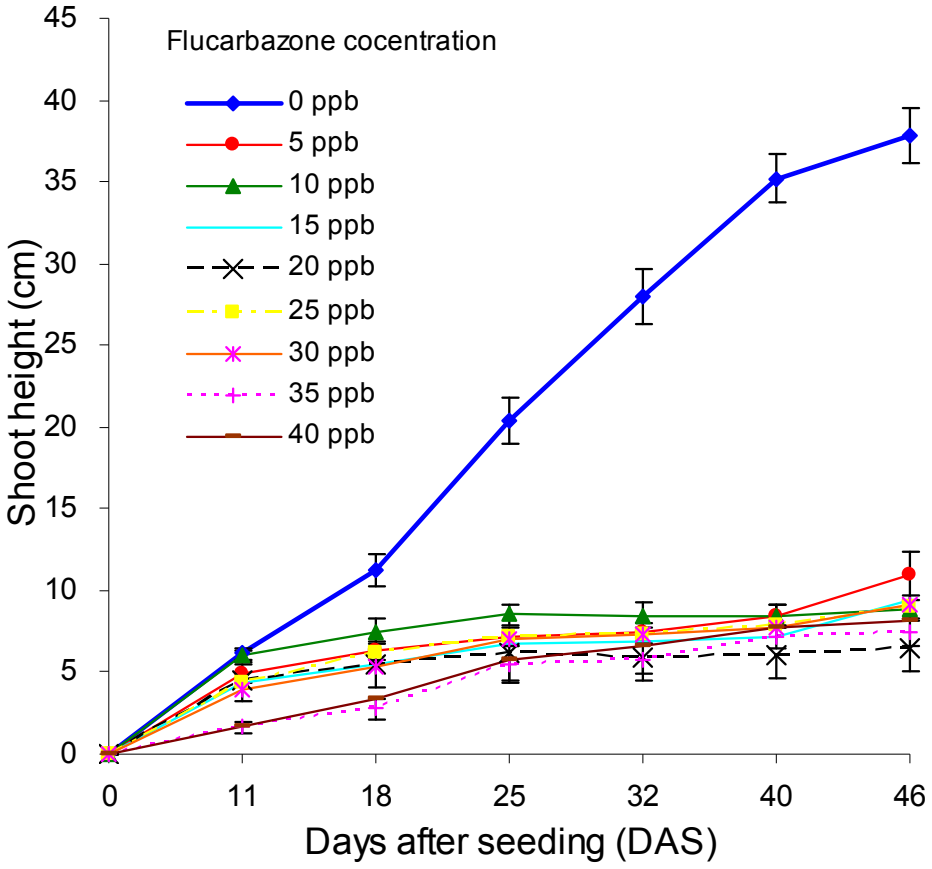

Figure 3.1 Impact of flucarbazone, applied at varying concentrations, on shoot height of field pea grown in a growth chamber. Error bars represent standard error.

Figure 3.2 Field pea leaves and tendrils at the time of harvest: (A) Control and (B) 20 µg kg^{-1} flucarbazone-added treatment. Recommended field application rate of flucarbazone is 30 g a.i. ha^{-1}, which approximately converts to 20 µg kg^{-1}.

As noted above, the control plants were nearly four times taller, on average, than any of the treated plants at the time of harvest, while there were no significant height differences among the treated plants at the time of harvest ($P < 0.05$; Fig. 3.1). This trend in height difference also was reflected in the dry mass of shoots and pods where control plants had a significantly greater above-ground biomass than any of the treated plants ($P < 0.05$); however, there was no statistically detectable difference in the above-ground biomass among the treated plants (Fig. 3.3). Similarly, root biomass of the control plants was nearly three times greater than any of the treated plants, and there was no statistically significant difference among the root biomass of the treated plants according to the ANOVA ($P < 0.05$; Fig. 3.4). When shoot and root biomass were combined to be analyzed as total biomass, the same trend was observed where control plants had a significantly greater total biomass than any of the treated plants ($P < 0.05$); however, there were no statistically detectable differences in the total biomass among the treated plants (Fig. 3.5). Interestingly, the increase in flucarbazone application rates from 5 μg kg^{-1} (lowest) to 40 μg kg^{-1} (highest) did not result in consistently observable trends in the shoot height and the shoot/root biomass (Fig. 3.1, 3.3, 3.4, 3.5), and all rates had a negative impact on these growth parameters.

3.3.2 Nodule number and weight

There was a notable difference in the number and the dry mass of nodules collected from control and flucarbazone-treated plants. On average control plants had seven times the number of nodules and nine times more nodule dry mass than the plants treated with the lowest amount of flucarbazone (5 μg kg^{-1}) (Fig. 3.6 and 3.7). Among the flucarbazone-treated plants, those treated with the lowest and the second lowest concentration of flucarbazone (5 μg kg^{-1} and 10 μg kg^{-1}, respectively) both had similarly small numbers and weights of nodules, which were significantly less than the control plants ($P < 0.05$; Fig. 3.6 and 3.7). Application of flucarbazone at rates greater than 15 μg kg^{-1} prevented any nodule formation (Fig. 3.6 and 3.7).

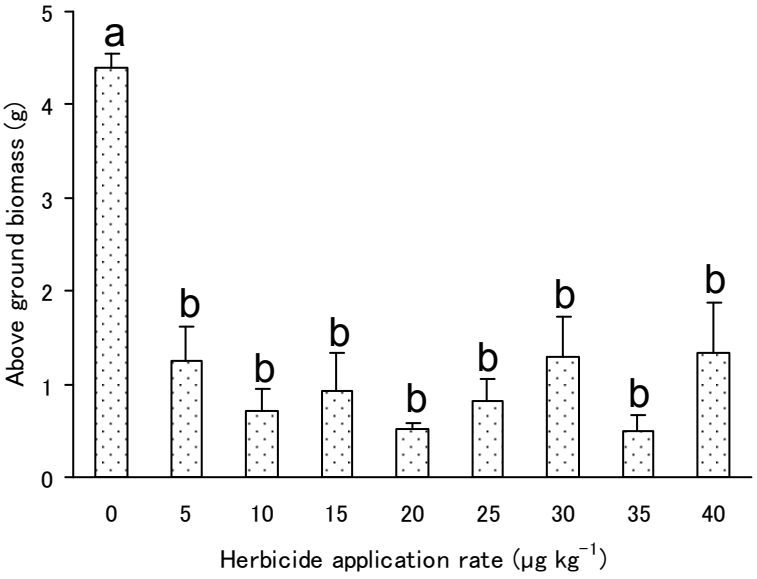

Figure 3.3 Influence of flucarbazone application rates on above-ground biomass (g per two plants) of field pea plants grown in a growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$. Recommended field application rate is 30 g a.i. ha^{-1}, which approximately converts to 20 μg kg^{-1}.

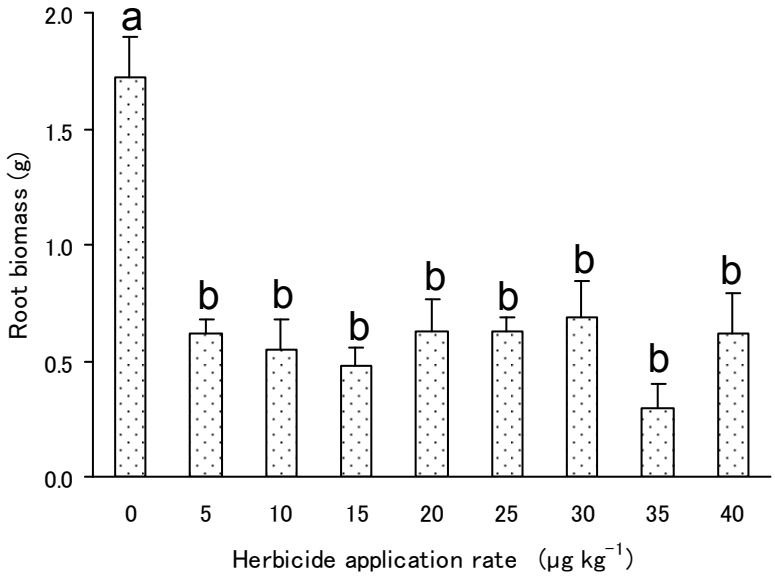

Figure 3.4 Influence of flucarbazone application rates on root biomass (g per two plants) of field pea plants grown in a growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$. Recommended field application rate is 30 g a.i. ha^{-1}, which is approximately 20 μg kg^{-1}.

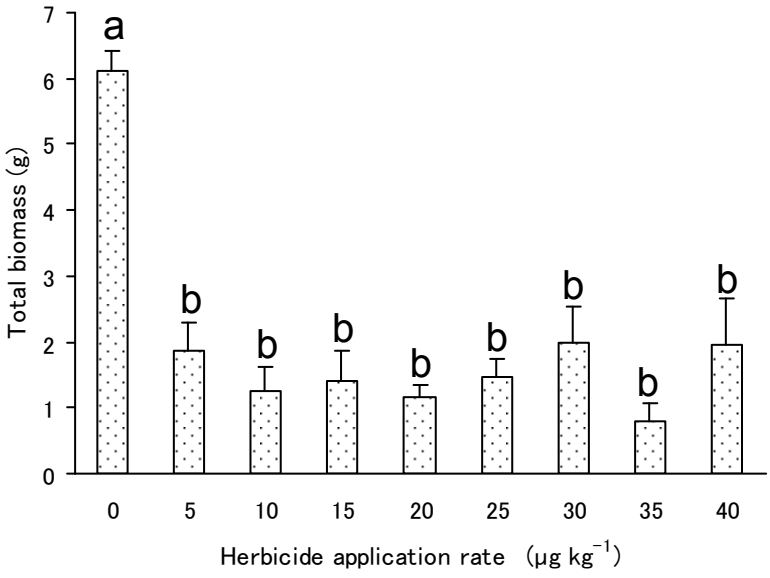

Figure 3.5 Influence of flucarbazone application rates on total biomass (g per two plants) of field pea plants grown in a growth chamber. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$. Recommended field application rate of flucarbazone is 30 g a.i. ha^{-1}, which approximately converts to 20 μg kg^{-1}.

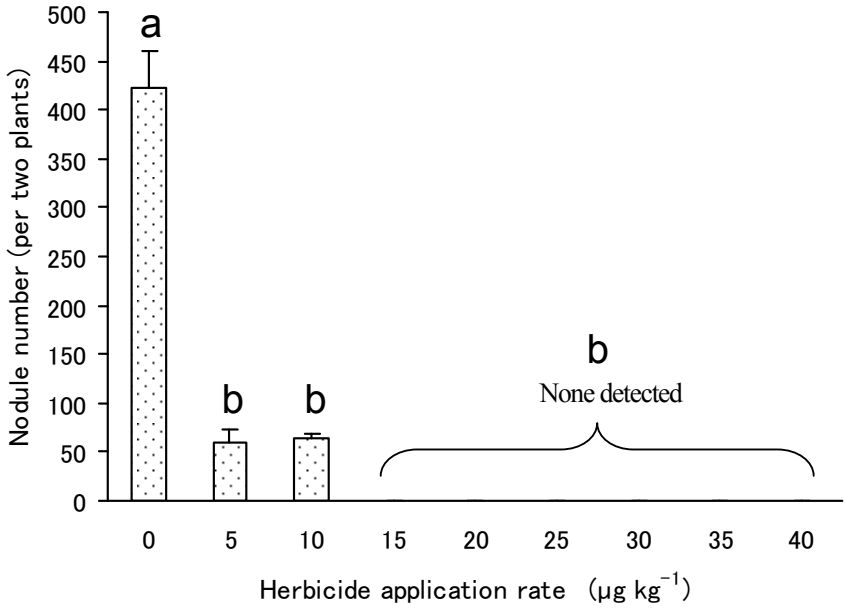

Figure 3.6 Influence of flucarbazone application rates on the number of nodules collected from field pea plants (per two plants) grown in a growth chamber, measured at 46 days after seeding. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

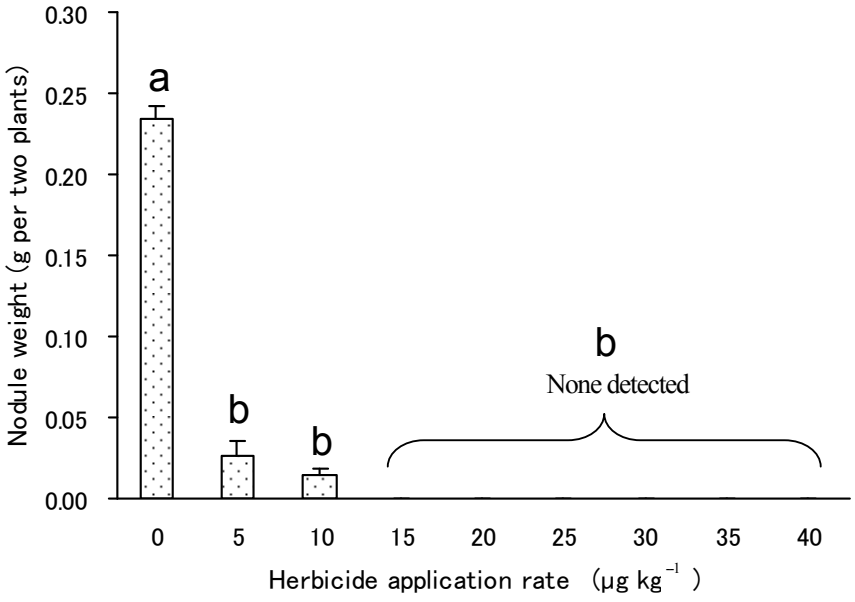

Figure 3.7 Influence of flucarbazone application rates on the nodule biomass (g per two plants) collected from field pea plants grown in a growth chamber, measured at 46 days after seeding. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

3.3.3 $\delta^{15}N$ and total N in above-ground biomass

Only the $\delta^{15}N$ of above-ground biomass obtained from the control plants had a negative value (‰), which was significantly less ($P < 0.05$) than any of the positive $\delta^{15}N$ values obtained from the flucarbazone-treated plants (Fig. 3.8). There were no significant differences in above-ground biomass $\delta^{15}N$ values among treated plants ($P < 0.05$), and the increase in flucarbazone application rates from 5 μg kg^{-1} (lowest) to 40 μg kg^{-1} (highest) did not generate a notable trend in the $\delta^{15}N$ values (Fig. 3.8).

The N concentration (μg mg^{-1}) of above-ground biomass was unique in that the average value from the control plants did not vary appreciably from any of the flucarbazone-treated plants (Fig. 3.9). According to the ANOVA ($P < 0.05$), there were statistically significant differences observed among the N concentration values of select treatments; however, their occurrence was random, and the differences did not correspond with incremental changes in herbicide application rates.

3.3.4 Nodule occupancy

The plants that formed nodules (0, 5, and 10 μg kg^{-1} flucarbazone-treated) were analyzed for their nodule occupants, and the proportion of successful nodule colonization by the inoculum strain was compared. The photographs of the agarose gels, visualizing PCR-IGS products, were used to visually compare banding patterns of rhizobial strains extracted from nodules to that of the reference inoculum strain. An example of the photographed agarose gel is shown in Fig. 3.10. Electrophoresis of PCR-IGS products revealed that the reference strain possessed one band whereas some strains isolated from nodules possessed multiple bands (Fig. 3.10). Also, the lengths of the PCR-IGS products were not identical for all strains tested which varied from approximately 600 to 1400 bp long.

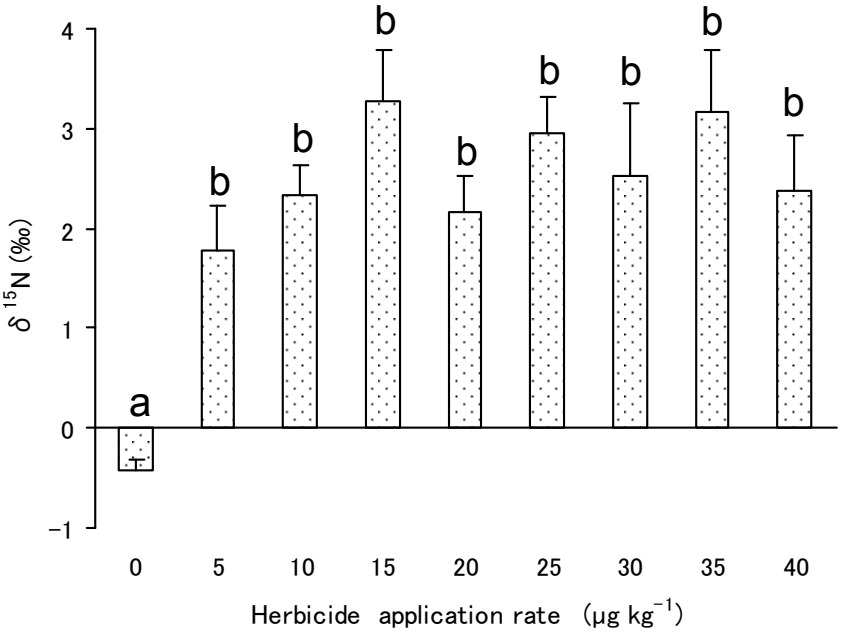

Figure 3.8 Influence of flucarbazone application rates on $\delta^{15}N$ (‰) of above-ground plant material obtained from a growth chamber experiment at 46 days after seeding. Error bars represent standard error. Bars with different letters are statistically different according to Tukey's test at $P < 0.05$.

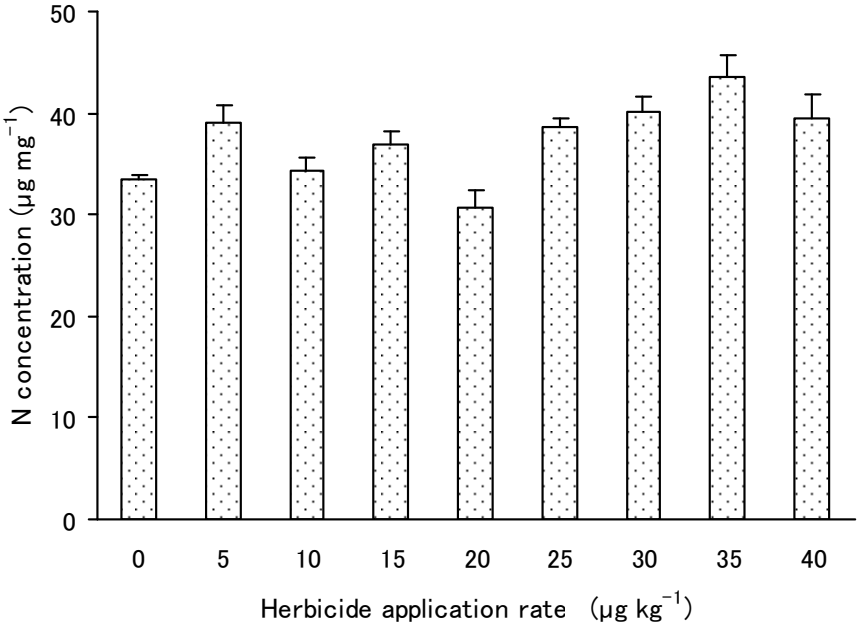

Figure 3.9 Influence of flucarbazone application rates on N concentration (μg mg^{-1}) of above-ground plant material obtained from a growth chamber experiment at 46 days after seeding. Error bars represent standard error. According to Tukey's test at $P < 0.05$, statistically significant differences were observed among the N concentrations of select treatments; however, their occurrence was random, and the differences did not correspond with incremental changes in herbicide rates.

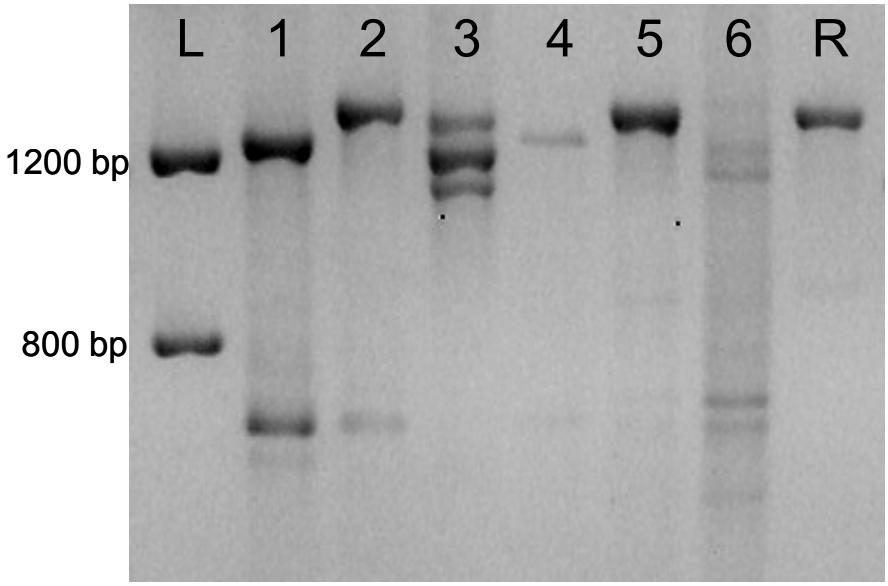

Figure 3.10 Banding patterns of PCR amplified IGS products ran on 2% agarose gel. Lane L: Low DNA Mass Ladder; Lane 1 to 6: *Rhizobium* strains extracted from crown nodules of control plants; Lane R: Reference inoculum strain.

The results from the visual examination of the agarose gel images were then reported as the proportion of successful nodulation by the inoculum strain (Table 3.1). There were no statistically detectable differences in the proportion of successful nodulation by the inoculum strain when nodules from each region (i.e., crown or distal) were compared separately across treatments (crown: $\chi^2 = 4.16$, $P = 0.13$; distal: $\chi^2 = 1.03$, $P = 0.60$), and the increasing flucarbazone application rates did not cause the inoculation success levels to have a notable trend (Table 3.1). Also, there were no significant differences in the nodulation success when sampling regions were compared within each herbicide application rate (i.e., crown vs. distal) (0 μg kg^{-1}: $\chi^2 = 3.02$, $P = 0.08$; 5 μg kg^{-1}: $\chi^2 = 1.42$, $P = 0.23$; and 10 μg kg^{-1}: $\chi^2 = 0.00$, $P = 1.00$).

Table 3.1 Influence of flucarbazone application on the proportion of successful nodule colonization by inoculum strain. Nodules were collected from field pea plants grown in a growth chamber, measured at 46 days after seeding.

	Flucarbazone application rate		
	0 µg kg^{-1}	5 µg kg^{-1}	10 µg kg^{-1}
Crown Average	4/6	2/6	3/6
Distal Average	2/6	3/6	3/6

* For each treatment, four replicated pots were present with each pot containing 2 plants. From each replicated pot, six nodules were analyzed for each region (crown or distal), and the presence of the inoculum strain was recorded in terms of a fraction as in "X/6" where X = number of nodules containing an inoculum strain.

** The fractional values from the four replicated plots were averaged, rounded to the nearest whole number, and presented in the table.

3.4 Discussion

3.4.1 Shoot growth and nodule formation

The results from the herbicide-spiked growth chamber study clearly indicated the susceptibility of field pea to the presence of flucarbazone in the soil, demonstrated by the significant reduction in the shoot and root development, nodule production, and N_2 fixation induced by the herbicide addition (Fig. 3.1 to 3.7). There are numerous reports in the literature of reduction in plant/nodule production and N_2 fixation induced by various AHAS-inhibiting herbicides in different leguminous plants (Gonzalez et al., 1996; Royuela et al., 2000; Anderson et al., 2004; Zawoznik and Tomaro, 2005). Unfortunately, published data specific to the influence of flucarbazone on field pea growth, nodule production, and N_2 fixation is not available at the time of writing; however, some other AHAS inhibitors have been reported to influence these parameters in peas.

Gaston et al. (2002) transplanted 12-day-old field pea plants into a hydroponic system with nutrient solution, containing 140 times the recommended field application rate of an AHAS inhibitor (imazethapyr; IM), to study the change in shoot and root lengths. The resultant shoot growth pattern reported by Gaston et al. (2002) was very similar to those from the current study where the control plants continued to grow until harvest (7 d after IM treatment), while the shoot growth was arrested in IM-treated plants 3 d after the herbicide addition. Interestingly, the AHAS inhibitors prevented elongation of shoots and roots, yet the plants continued to live until harvest in the current study and in the experiment conducted by Gaston et al. (2002).

Blair (1988) reviewed the visible symptoms of AHAS inhibitors on plants and reported that plants treated with sulfonylureas usually died slowly, and older plants decreased in susceptibility to the herbicide while some recovered from the inhibition in

growth. Although stunted and malformed, these plants produced normal leaves, flowered and produced seeds (Blair and Martin, 1988).

Gonzalez et al. (1996) conducted a 4-wk growth chamber study with field pea and reported that while the application of 3.5 times the recommended field application rate of IM reduced nodulation by 45%, the application of IM at above 3.5 times the recommended field application rate nearly eliminated the nodule formation. Similarly, in the current study, the treatment of plants with the lowest and the second lowest concentration of flucarbazone (5 μg kg^{-1} and 10 μg kg^{-1}, respectively) reduced the nodulation by approximately 85%, and the application of flucarbazone at 15 μg kg^{-1} or more eliminated nodule formation. These findings suggest the presence of a threshold herbicide concentration for a given herbicide with which nodulation is abolished when it is exceeded. Published data is not available to confirm the presence of such a threshold level for field pea treated with flucarbazone.

3.4.2 N fixation assessed by $\delta^{15}N$ and total N in above-ground biomass

Because of isotope discrimination caused by biological and chemical processes, most soils have slightly higher ^{15}N abundance than the atmosphere (Amarger et al., 1979). As a result of such a difference in ^{15}N abundance between soil and atmospheric N_2, N_2-fixing plants have been found to have lower ^{15}N enrichment than non-fixing ones (Amarger et al., 1979). Since the shoot $\delta^{15}N$ (‰) values are presented in terms of the relative ^{15}N abundance of a given sample to that of the atmosphere, those plants with positive shoot $\delta^{15}N$ values are thought to have utilized relatively more N from the soil than the control plants which primarily obtained N from the atmosphere via symbiotic N_2 fixation. Since isotopic fractionation has been reported to occur with some legumes during N_2 fixation by the legume-rhizobia symbiosis with a preference for ^{14}N over ^{15}N

(Yoneyama et al., 1986), it is not unusual for actively N_2-fixing plants to have negative shoot $\delta^{15}N$ values.

Since the flucarbazone added plants had practically no nodulation (Fig. 3.6) and the tissue N concentrations were more or less comparable over the range of herbicide application without any notable trends (Fig. 3.9), it is safe to assume that the control plants met their N demand via symbiotic N_2-fixation, whereas the flucarbazone-treated plants satisfied N requirements by taking up N from the soil. The growth arrest caused by the herbicide also may have contributed in the maintenance of N levels in flucarbazone-treated plants similar to control plants. Published data specific to the influence of flucarbazone on N fixation in field pea was not available at the time of writing.

Zawoznik (2005) reported that the treatment of soybean with AHAS inhibitor (chlorimuron-ethyl) reduced the number of nodules by 38% but shoot N content remained constant. Unlike the present study, however, the herbicide-treated plants still produced a small number of nodules, and this led Zawoznik (2005) to speculate that the herbicide-treated soybean plants formed fewer but more active nodules. Shoot $\delta^{15}N$ values of control and treated plants were not presented by Zawoznik (2005) to analyze the potential N source difference.

3.4.3 Influence of flucarbazone on nodulation success by an inoculum strain

The current study found no difference in the proportion of successful nodule occupancy by the inoculum strain among different treatments of flucarbazone (0, 5, 10 μg kg^{-1}), and between crown and distal regions of a given treatment (Table 3.1). Also, the application of flucarbazone at rates greater than 15 μg kg^{-1} prevented any nodule formation (Fig. 3.4). In the absence of published data on the influence of AHAS inhibitors on nodule occupancy, an attempt is made to speculate how flucarbazone

application would have influenced rhizobia and/or the host plant to produce such results. Because AHAS inhibitors can affect both rhizobia and the plant, it is necessary to analyze the potential herbicidal influence on both symbiotic partners.

Multiple reports are present on the apparent resistance of rhizobia to high-concentrations of various AHAS-inhibiting herbicides. Gonzalez et al. (1996) reported that imazethapyr doses 700 times the recommended field application rates did not influence the growth and the nodulation ability of *Rhizobium leguminosarum* biovar *viceae* pure culture grown in complex and defined medium. Similarly, the growth of soybean rhizobia (*Bradyrhizobium japonicum*) was not affected when 150 times the recommended field application rates of chlorimuron-ethyl was added to the pure culture (Zawoznik and Tomaro, 2005). Furthermore, Anderson et al. (2004) reported that the addition of chlorsulfuron at double the recommended field application rate did not influence chickpea rhizobia (*Mesorhizobium ciceri*) grown in medium containing no external source of amino acids. The results from these studies suggest that rhizobial growth is generally unaffected by most AHAS-inhibiting herbicides, except at levels far exceeding those encountered in the field. In this study, nodulation was eliminated at 15 $\mu g\ kg^{-1}$ which is approximately 65% of the field recommended application rate. Given the apparent tolerance of rhizobia to various AHAS inhibitors indicated in the literature, 15 $\mu g\ kg^{-1}$ of flucarbazone did not likely hinder the multiplication of rhizobia in soil, but apparently influenced the ability to nodulate.

Interestingly, Anderson et al. (2004) reported in the aforementioned study of chickpea rhizobia that there was a reduction in number and weight of chickpea nodules when rhizobia were pre-exposed to chlorsulfuron at double the recommended field application rate. This means that the nodulation ability of rhizobia can be affected without any apparent effect on its growth characteristics. This is possible when the *nod* genes involved in the nodulation process are part of an extrachromosomal genetic

element nonessential for growth (i.e., plasmids), and not part of the bacterial chromosome (Gonzalez et al., 1996). Anderson et al. (2004) designed the experiment so that AHAS inhibitor was only present during the pre-exposure of rhizobia to the herbicide in a pure culture, and the herbicide was absent from any other stages of chickpea production. This led Anderson et al. (2004) to speculate that the reduction in nodulation resulted from an effect of the herbicide on the nodule formation process. Thus, it is possible that 15 $\mu g\ kg^{-1}$ of flucarbazone hindered the nodulation ability of all of the rhizobia present in soil to cause elimination of nodulation at that flucarbazone application rate. Having speculated as such, it is difficult to hypothesize, given the lack of information, if an AHAS inhibitor would affect one strain of rhizobia or a large group of related strains in their nodule formation process. Depending on the scope of influence of a given AHAS inhibitor, it can selectively influence the success of nodule occupancy by a certain strain or it can influence a large group of compatible rhizobia to affect nodulation in general.

Aside from its potential influence on rhizobia, AHAS inhibitors can influence the root biomass and its surface area to which rhizobia attach themselves when initiating nodulation. The herbicide influence on the root development can affect rhizobial infection of root hairs because the site of infection is restricted to actively growing hairs and the infection period is transitory (Gonzalez et al., 1996). Such change in physiological development of roots would likely influence nodulation by all compatible rhizobia present in soil regardless of their strains, and affect nodulation in general. In addition to the herbicidal influence on rhizobial nodulation capabilities, the influence of flucarbazone on root development may also explain the elimination of nodulation in the present study although root hair development was not examined.

The possibility of herbicidal interference of chemical signalling between rhizobia and host plant also has been suggested (Fox et al., 2007); however, the author is

not aware of published data on the topic specific to AHAS inhibitors and field pea. Therefore, it is not possible to speculate if herbicidal interference of communication between the symbiotic partners would be strain specific to impact nodulation success of a certain strain or if it would influence a large group of compatible rhizobia to affect nodulation in general. Nonetheless, the current study found that the flucarbazone application at 5 and 10 μg kg^{-1} significantly reduced the nodule count without influencing the nodulation success ratio by the inoculum strain. This may suggest that flucarbazone equally influences all rhizobial strains, rather than being selectively discriminatory against the inoculum strain tested in this study.

3.5 Conclusions

The findings from this study demonstrated the susceptibility of field pea to the presence of flucarbazone in soil. Given high enough concentrations of residual flucarbazone remaining in soil, the herbicide has the potential not only to hinder the proper physiological development of the plant, but also to eliminate nodulation altogether. Given the lack of published data on the influence of AHAS inhibitors on nodulation success however, it was not possible to validate findings on nodule occupancy from the present study. Even though an attempt was made to confirm the results based on the data from related studies, it was a challenging task because AHAS herbicides can potentially influence both symbiotic partners at various stages of nodule formation. Nonetheless, it is clear that if the environmental conditions are such that the concentration of residual flucarbazone in soil approaches 5 μg kg^{-1} (25% of recommended field application rates), a producer would likely notice a significant reduction in plant growth, nodulation, and N benefit of growing field pea. These findings from the growth chamber study raise questions as to whether or not similar results would be obtained in the field conditions where actual field pea production takes place.

4 THE EFFECT OF FLUCARBAZONE APPLICATION ON GROWTH PARAMETERS, NITROGEN FIXATION, AND NODULE OCCUPANCY OF FIELD PEA: FIELD EXPERIMENT

4.1 Introduction

In western Canada the chief cereal crop, wheat, is often followed with annual grain legumes such as field pea (*Pisum sativum* L.), chickpea (*Cicer arietium* L.), and lentil (*Lens esculenta* L.) in a sequential rotation. This type of crop rotation is beneficial in conservation and replenishment of soil N based on the ability of the leguminous plant to fix atmospheric N_2 through symbiotic association with effective strains of *Rhizobium* bacteria (Baldock et al., 1981; Wright, 1990; Beckie and Brandt, 1997; Przednowek et al., 2004) (Beckie and Brandt, 1997; Przednowek et al., 2004; Wright, 1990).

Certain herbicides used during wheat production can persist in soil longer than desired, and injure subsequently planted legumes (Moyer et al., 1990); however, a comprehensive study has not been conducted to fully examine the potential effects of a residual herbicide on the symbiotic association between the host legume and the *Rhizobium* bacteria. Based on the sensitivity of symbiotic partners to changes in many environmental factors, agrochemical residues in the soil holds great potential to influence inoculation success, N_2 fixation, and yield of grain legumes.

Given their residual properties (Colborn and Short, 1999), severe plant toxicity at low concentrations (Santel et al., 1999), and their potential ability to influence both host plant and the bacterial symbiotic partner (Duggleby and Pang, 2000), acetohydroxyacid synthase (AHAS)-inhibiting herbicides are of particular concern when

planning crop rotations. To examine the potential of an AHAS inhibitor to cause carryover injury in field pea cropped following wheat, a field experiment was conducted with one of the AHAS-inhibiting herbicides (flucarbazone-sodium) commonly used in western Canada in wheat production.

It was established in our previous growth chamber experiment (Chapter 3) that at a high enough concentration (i.e., 5 μg kg^{-1} of freshly added flucarbazone; approximately 25% of recommended field application rates), flucarbazone in soil has the potential to hinder proper development of field pea and restrict nodule development. By studying the potential influence of field-aged, one-year-old residual flucarbazone on field pea, the present field work was intended to elucidate whether flucarbazone applied at the field recommended rate during wheat production would persist for 1 yr at high enough concentrations (as either non-degraded flucarbazone or in the form of metabolites) to cause the type of negative impact on plant growth and nodule development as was observed in the growth chamber experiment. Also, a possibility was recognized that the proportion of nodules occupied by the inoculum rhizobial strains may vary among different flucarbazone treatments without affecting any of the plant and nodule development parameters. Thus, an attempt was made to genetically identify the origin of nodule occupants using a molecular microbiological method.

4.2 Materials and Methods

4.2.1 Description of field sites and soil characteristics

Field experiments were conducted in the spring/summer of 2005 and 2006 at the University of Saskatchewan Goodale Crop Research Farm, Saskatchewan (SW3-36-4-W/3) and the Agriculture and Agri-Food Canada Research Station at Beaverlodge, Alberta (NW36-71-10-W/6). In order to simulate flucarbazone carryover during crop rotation, wheat was grown in 2005 (year 1), and was treated with three rates of

flucarbazone-sodium (0, 20, 30 g a.i. ha^{-1}) to control weeds in wheat, where the recommended field rate is 30 g a.i. ha^{-1} (≈ 20 μg kg^{-1}). The control plots that received no flucarbazone were treated with non-residual herbicides containing no AHAS inhibitor (i.e., bromoxynil and clodinafop mix) at the field recommended application rates to control weeds. At each site, all treatments were replicated four times, and arranged in a randomized complete block design.

Prior to seeding pea in 2006 (year 2), soil samples (0- to 10-cm depth) were collected from each replicated plot with a shovel, and bulked together to obtain a composite sample for each treatment at each location. At each replicated plot, three to five sampling locations were randomly selected, avoiding the perimeters of the plot. Each composite soil sample was air-dried in the laboratory, ground to pass through a 2-mm sieve, and stored for analyses. Composite soil samples of control plots were analyzed to obtain general physical and chemical characteristics of the soil at each site (Table 4.1). Soil texture analysis was conducted with a laser diffraction particle size analyzer according to the manufacturer's instructions (Horiba Instruments, Inc., Irvine, CA). Percent organic carbon (OC) contents were determined using a LECO CNS-2000 furnace (LECO Corporation, St. Josheph, MI) at 840 ºC (Wang and Anderson, 1998). Soil pH was measured with a pH meter in a 1:2 soil:water suspension (McLean, 1982). Field capacity (FC) moisture content of the soil was approximated according to Eliason et al. (2004). Both ammonium (NH_4) and nitrate (NO_3) concentrations in soil was measured by using a 2M KCl soil extraction method as described by Page (1982).

Table 4.1 Soil characteristics in the upper 10 cm of the soil profile at trial sites: Goodale SK and Beaverlodge, AB

Site	Soil Classification	Texture	Sand	Silt	Clay	OC†	pH	FC‡	NH_4	NO_3
			----------%----------						µg/ g soil	µg/ g soil
Goodale, SK	Dk Br Chernozem	Sandy Clay Loam	65	12	23	2.2	5.9	18	8	57
Beaverlodge, AB	Dk Grey Luvisol	Clay	25	24	51	3.9	5.5	29	6	89

† Percent organic carbon

‡ Percent moisture (w/w) at field capacity

4.2.2 Pea seeding and inoculation

In year 2, the same field plots at Goodale, SK and Beaverlodge, AB were seeded to field pea (*Pisum sativum* cv CDC Mozart) which was pre-treated with a commercial peat inoculant (NitraStik-C®; EMD Crop BioScience, WI) containing two strains of *R. leguminosarum* bv. *viceae*. The pure cultures of each strain were generously provided by EMD Crop BioScience, and designated as reference strains R_1 and R_2. The product label for NitraStik-C® states that a gram of the product delivers a minimum of 500 million viable cells of *R. leguminosarum* bv. *viceae* (EMD Crop BioScience, 2007). Given the rate of inoculant used for this experiment (4g per kg of seeds) and the approximate average weight of a pea seed (0.2 g), each seed would have, in theory, received at least 4.0×10^5 viable rhizobial cells. The information was not available on the relative proportion of the two strains R_1 and R_2 present in the inoculant.

4.2.3 Acetylene reduction assay

When the field peas were at flowering to pod formation stage, random samples of three plants from each treatment plot were collectively tested for nitrogenase activity according to the acetylene reduction assay (ARA) (Hardy et al., 1973). Upon excavation of the plants, excess soil was gently removed from roots, shoots were cut off, and roots

were placed in a gas tight 1 L Mason jar equipped with a rubber septum installed in the lid. One hundred millilitres of air was replaced with an equal volume of acetylene (C_2H_2) using a syringe, and the jar was buried in soil for incubation. During the 20-min incubation period, the jar was dug up every 5 min to be shaken gently to ensure nodule exposure to C_2H_2. After incubation, 10 mL of the gas sample was removed from the jar with a syringe, and placed into an evacuated gas tight test tube (BD Vacutainer®) through a septum installed in the lid. A total of twelve gas samples (i.e., 0, 20, 30 g a.i. ha^{-1} flucarbazone treated plots x 4 replicates) were brought back to the laboratory, and analyzed on a gas chromatograph (GC; Hewlett-Packard 5890A) fitted with a flame ionization detector (FID) for the concentration of nitrogenase-reduced acetylene (i.e., ethylene: C_2H_4). Chromatographic separation was carried out on a 1.8m Porapak™ Q column (80/100 mesh), and the instrument was run at 60 °C with nitrogen as the carrier gas at flow rate of 40cc min^{-1}.

4.2.4 Observation of shoot and root biomass and nodule formation

After the ARA was conducted in the field, the harvested shoots and roots were brought back to the laboratory for the determination of plant biomass and nodulation. Root samples were washed clean with a gentle stream of tap water prior to nodule removal, and then nodule numbers were recorded on three plants per plot. Shoots, roots, and detached nodules were oven dried (shoots and roots = 72 h at 45 °C; nodules = 24 h at 60 °C), and weights were recorded for each plot.

4.2.5 Observation of N concentration, and ^{15}N accumulation

At pea seed maturity in year 2, above-ground biomass (shoot and seeds) was harvested by hand from each plot by randomly selecting $1m^2$ area (containing four 1-m crop rows) in the middle of each plot. The plant sample from each plot was individually

bagged, air-dried, weighed, threshed, and seed yield recorded. Shoot samples were roughly ground in a grinder, re-ground in a rotating ball-bearing mill into fine powder, and analyzed for N content (%) using a LECO CNS-2000 furnace (LECO Corporation, St. Josheph, MI). Subsamples (1 ± 0.05 mg) of the powderized shoot samples were also analysed for natural abundance (δ) of the stable isotope pair $^{15}N/^{14}N$ as described under Section 3.2.3.

4.2.6 Nodule occupancy analysis

At the same time as when in-field ARA was conducted at flowering to pod formation stage, a separate set of field pea samples were collected for nodule occupancy analysis. Three plants were randomly selected from each replicated plot, excavated with a shovel, and the whole plant brought back to the laboratory with soil attached to the roots. In the laboratory the roots were washed clean of soil using a gentle stream of tap water, and the plant was air-dried at room temperature. Once a constant weight was achieved, nodules were removed from roots, and grouped into crown and distal regions, as described under Section 3.2.4.1. DNA extraction and PCR amplification were conducted on nodule samples as previously described under Sections 3.2.4.1 and 3.2.4.2, respectively. When it was necessary to PCR-amplify the inoculum strains as a reference, their pure culture bacterial cell suspensions were used as a template.

4.2.7 Analysis of nodule occupancy data

At each field site, four replicated plots were present for each treatment. From the plant samples collected to represent each replicated plot, six nodules were analyzed for each region (crown and distal), the presence of the inoculum strain was recorded in each nodule, and results are reported in terms of a fraction, e.g. “X/6” where X signifies the number of nodules containing an inoculum strain. To statistically analyze the differences

in the proportion of successful colonization by the inoculum rhizobial strains among the treatments, a contingency table similar to the example shown in Appendix B was constructed, and a Pearson's chi-square (χ^2) test was conducted in SPSS (SPSS Inc, 2006).

4.2.8 Verification of the nodule occupancy analysis

After the visual analysis of agarose gel photographs, a select number of sample nodule occupants and the reference rhizobial strains were analyzed for their 16S-23S rDNA intergenic spacer (IGS) nucleotide sequences. Sequencing was conducted to verify the accuracy of the visual classification of nodule occupants on the basis of banding patterns (i.e., matching or non-matching to the reference strain), and to confirm that PCR-IGS products of indistinguishable band mobility are indeed identical in the IGS gene sequences. The verification was jointly conducted for the growth chamber study (Chapter 3) and the field study.

4.2.8.1 Sample preparation for nucleotide sequencing

Select rhizobial DNA extracts from sample nodules and the reference inoculant strains were subjected to PCR reaction (40 µL reaction volume per tube x 4 tubes = 160 µL IGS-PCR product per nodule sample) as described under Section 3.2.4.2. The PCR amplified IGS product (PCR-IGS) of each sample was combined in a 1.5 mL Eppendorf tube, 16 µL of 3*M* sodium acetate (pH 5.2) and 440 µL of 95% ethanol were then added to precipitate DNA overnight at – 20 ºC. The precipitated DNA was centrifuged for 15 min at 4 ºC (13,000 rpm), and supernatant was discarded to leave the DNA pellet on the bottom of the tube. To wash the tube and the DNA pellet, 70% ethanol (500 µL) was added to each tube, gently inverted, centrifuged for 5 min at room temperature (13,000

rpm), and the ethanol was discarded. After washing the DNA twice in this manner, excess ethanol was allowed to evaporate in a bio-safety cabinet. The cleaned DNA pellet was dissolved in 30 µL of Tris ETDA buffer (pH 8.0), then 1 µL of the dissolved product was quantified for the DNA concentration with a 1% (w/v) agarose gel and a dilution series of a Low DNA Mass^{TM} Ladder (Invitrogen, California, USA).

After quantification, the remaining PCR-IGS products were run on 1% agarose gel. A select number of single bands produced by the sample nodules and the reference inoculum strains were excised under the UV illumination, and DNA was eluted from the excised gel and purified using Geneclean II kit (MP Biomedicals, California, USA). The National Research Council – Plant Biotechnology Institute DNA Sequencing Lab (Saskatoon, Canada) performed the sequencing reactions using the primer FGPS1490. The sequencing results were compared against the microbial nucleotide sequence database to determine the most closely related strain using the National Center for Biotechnology Information (NCBI) online standard BLAST (Basic Local Alignment Search Tool) program (http://www.ncbi.nlm.nih.gov/).

4.2.8.2 Selection of banding patterns to be sequenced

The agarose gel electrophoresis of PCR-IGS products obtained from nodule samples and reference inoculum strain produced unique sets of banding patterns for the growth chamber experiment (Fig. 3.10). As reported later in this chapter (Section 4.3.3), the PCR-IGS products of nodule samples and reference strains from the present field experiment also produced unique banding patterns upon electrophoresis which were distinguishable from each other. Excluding the ones that were visually quite different from the reference strains, five banding patterns were identified in the sample nodules as containing either ‘similar’ or ‘identical’ bands as those of the reference strains based on

their migratory distance. These patterns were classified as A, B, C, R'_1, and R'_2 to signify that the bands A to C were similar to (but visually distinguishable from) one of the reference strains while the bands R'_1, and R'_2 were matching to (and visually indistinguishable from) the reference strains R_1 and R_2, respectively. For each pattern, three representative bands were excised from the agarose gel to have the PCR-IGS products of each pattern sequenced in triplicates as described in the previous section.

4.2.9 Statistical analysis

Except for those from molecular analysis of nodule occupancy, data were presented as means and standard errors, and the differences between treatments were assessed using one-way ANOVA with post hoc analysis (Tukey's test) using SPSS (version 15.0.1; SPSS, Chicago, IL). The data from molecular analysis of nodule occupancy were expressed in the form of a contingency table, and Pearson's chi-square (χ^2) test was conducted using SPSS (version 15.0.1; SPSS, Chicago, IL).

4.3 Results

4.3.1 Weather

During the 2005 growing season (year 1) when flucarbazone was applied in wheat at both field sites, Goodale had 83% more precipitation than the 30-yr average between May and September, while Beaverlodge received 17% less precipitation than the 30-yr average for the same period (Table 4.2). Similarly, during the 2006 growing season (year 2) when field pea was grown, Goodale had 70% more precipitation than the 30-yr average between May and September, while Beaverlodge received 32% less precipitation than the 30-yr average for the same period.

Table 4.2 Monthly precipitation (mm) for the two growing seasons at field trial locations: Goodale, SK and Beaverlodge, AB.

	2005					
	May	June	July	Aug.	Sept.	Total
Goodale, SK†	27.6	173.2	57.0	84.0	92.4	434.2
Beaverlodge, AB‡	52.4	71.4	65.4	49.2	17.2	255.6
	2006					
	May	June	July	Aug.	Sept.	Total
Goodale, SK†	58.2	110.8	45.8	35.4	125.2	375.4
Beaverlodge, AB‡	59.6	18.8	59.8	10.0	22.8	171.0

* 30 yr long term annual precipitation average (1970 – 2001) for Goodale, SK (244.3 mm) and Beaverlodge, AB (317.7 mm).

† Environment Canada Kernen weather station, SK

‡ Environment Canada Beaverlodge weather station, AB

4.3.2 Plant growth and nodulation

According to the ANOVA ($P < 0.05$), the use of various rates of flucarbazone in year 1 (0, 20, and 30 g a.i. ha^{-1}) did not cause statistically significant differences in any of the parameters measured in year 2 at either field site (Goodale, SK and Beaverlodge, AB). The parameters measured included: shoot and root dry biomass, seed yields, nitrogenase activity (ARA), nodule number and weight, shoot N concentration, and shoot $\delta^{15}N$ (Fig. 4.1.a to h and 4.2.a to h).

At Goodale, data suggested an inverse relationship between the incremental herbicide application rates and the average values of shoot weight and total nodule number (Fig. 4.1.a and e). Similarly, at Beaverlodge, data suggested an inverse relationship between the incremental herbicide application rates and the average values of seed yield (Fig. 4.2.c).

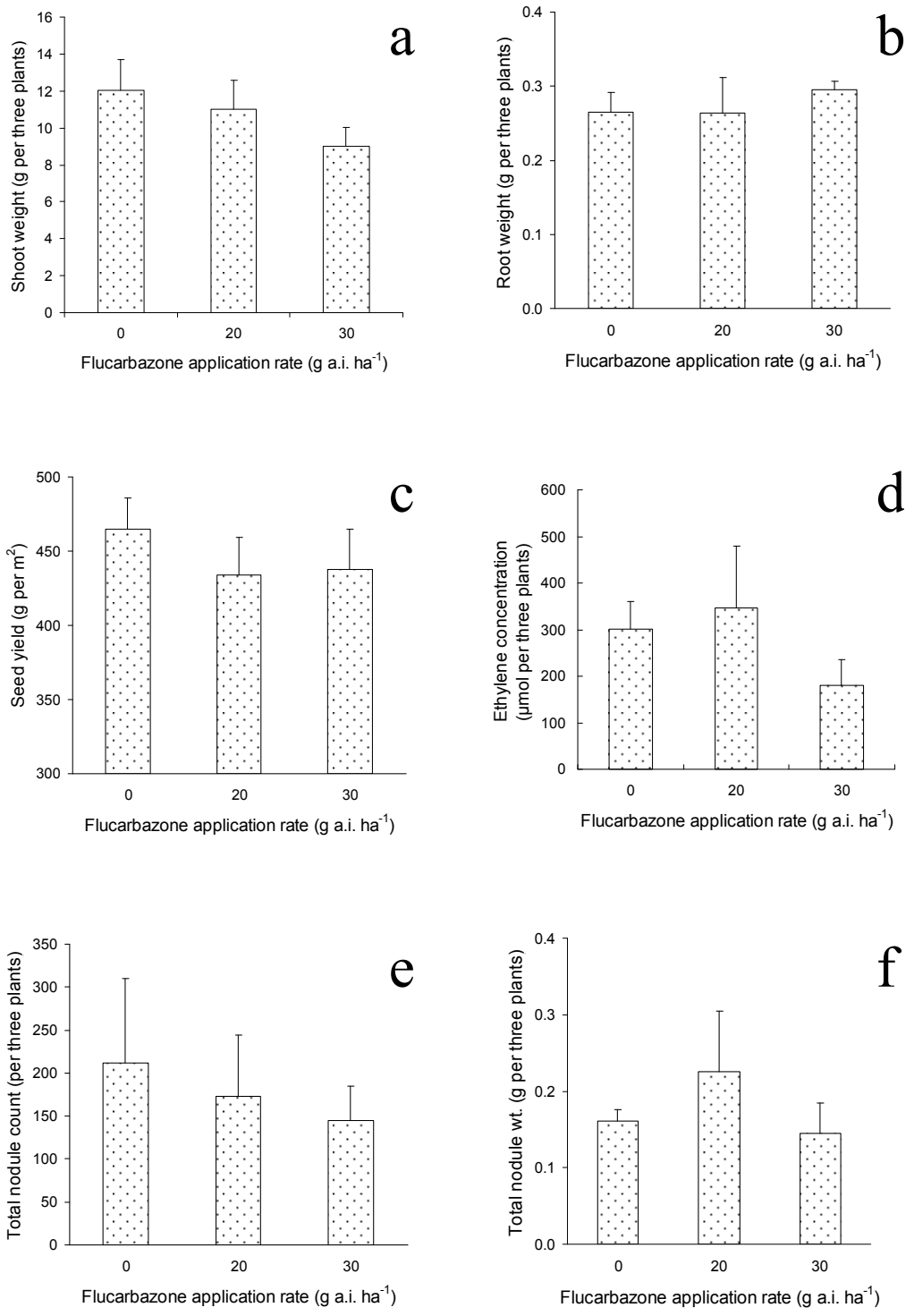

Figure 4.1 a to f. Impact of flucarbazone application on: shoot weight (a), root weight (b), seed yield (c), nitrogenase activity measured by acetylene reduction assay (d), total nodule number (e), and total nodule weight (f) of field pea grown at Goodale, SK. Error bars represent standard error. Differences in the reported mean values of each parameter among flucarbazone application rates were not statistically significant according to Tukey's test at $P < 0.05$.

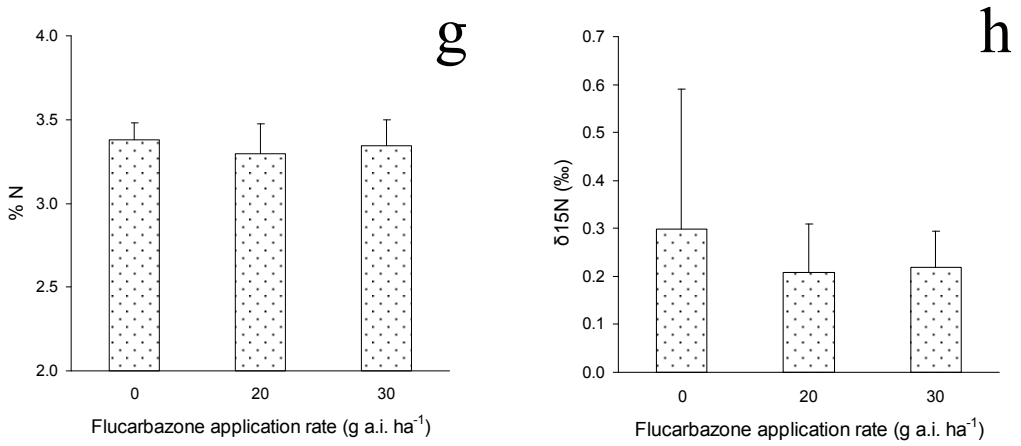

Figure 4.1 (continued) g to h. Impact of flucarbazone application on: shoot N concentration (g) and $\delta^{15}N$ (‰) of shoot (h) of field pea grown at Goodale, SK. Error bars represent standard error. Differences in the reported mean values of each parameter among flucarbazone application rates were not statistically significant according to Tukey's test at $P < 0.05$.

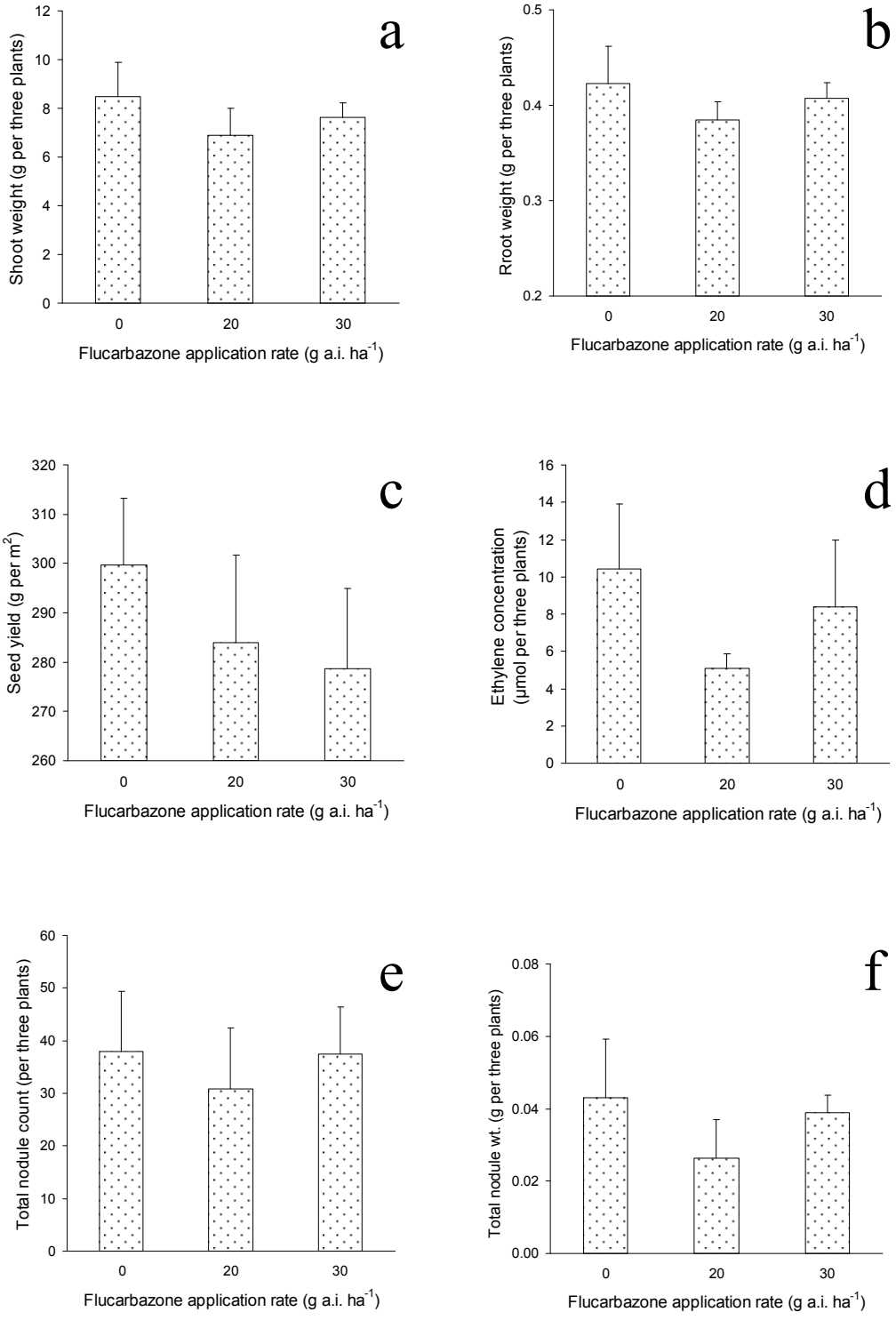

Figure 4.2 a to f. Impact of flucarbazone application on: shoot weight (a), root weight (b), seed yield (c), nitrogenase activity measured by acetylene reduction assay (d), total nodule number (e), and total nodule weight (f) of field pea grown at Beaverlodge, AB. Error bars represent standard error. Differences in the reported mean values of each parameter among flucarbazone application rates were not statistically significant according to Tukey's test at $P < 0.05$.

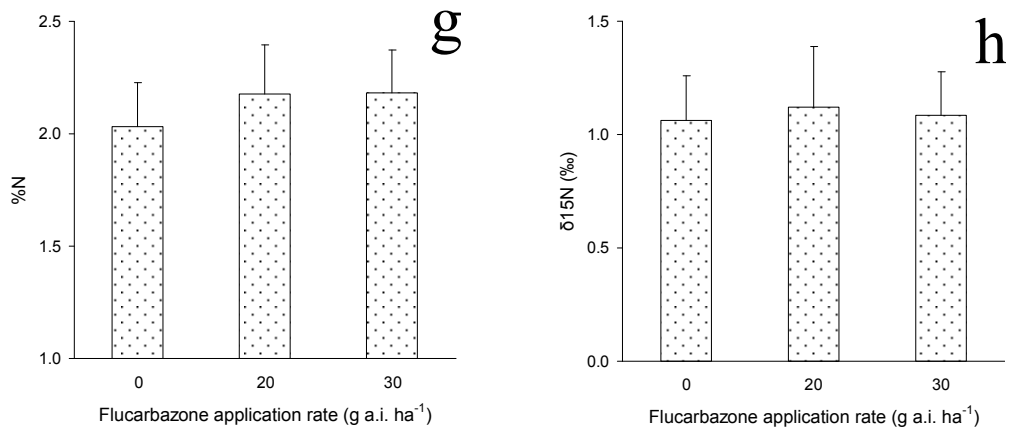

Figure 4.2 (continued) g to h Impact of flucarbazone application on: shoot N concentration (g) and $\delta^{15}N$ (‰) of shoot (h) of field pea grown at Beaverlodge, AB. Error bars represent standard error. Differences in the reported mean values of each parameter among flucarbazone application rates were not statistically significant according to Tukey's test at $P < 0.05$.

However, these relationships were not consistently observed at both locations. Moreover, the ANOVA failed to detect any statistically significant differences at $P < 0.05$. As for those parameters lacking any identifiable trends, the variation in their average values across treatments appeared random, and the differences did not correspond with incremental changes in herbicide application rates.

4.3.3 Nodule occupancy

Field pea harvested from the field sites with three different treatments (i.e., application of 0, 20, and 30 g a.i. ha^{-1} of flucarbazone in year 1 in wheat) were analyzed for their nodule occupants, and the proportion of successful nodule colonization by the inoculum rhizobial strains was compared. The photographs of the agarose gel electrophoresis, visualizing PCR-IGS products, were used to compare banding patterns of rhizobial strains extracted from nodule samples to those of the reference inoculum strains. An example of an agarose gel is shown in Fig. 4.3.

As in the growth chamber experiment (Chapter 3), electrophoresis of PCR-IGS products revealed that the reference inoculum strain R_1, as well as R_2, possessed one band whereas some strains isolated from nodules possessed multiple bands (Fig. 4.3). Also, the lengths of the PCR-IGS products were not identical for all strains tested which varied from approximately 600 to 1400 bp long. The results from the visual examination of the agarose gel images were then reported as the proportion of nodules successfully colonized by inoculum strains R_1 or R_2 (Table 4.3).

Statistically detectable differences in the proportion of successful nodulation by the inoculum strains were not found when nodules from each region (i.e., crown or distal) were compared across the treatments for Goodale (crown: $\chi^2 = 2.63$, $P = 0.27$; distal: $\chi^2 = 2.09$, $P = 0.35$) and Beaverlodge (crown: $\chi^2 = 1.57$, $P = 0.46$; distal: $\chi^2 = 0.38$, $P = 0.83$).

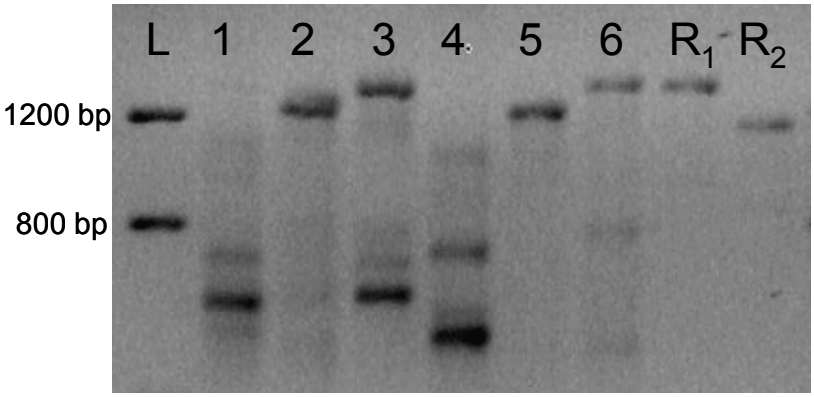

Figure 4.3 Banding patterns of PCR amplified IGS products ran on 2% agarose gel. Lane L: Low DNA Mass Ladder; Lane 1 to 6: *Rhizobium* strains extracted from crown nodules (Goodale, 20 g a.i. ha^{-1} treatment); Lane R_1 and R_2: Reference inoculum strains.

Table 4.3 Influence of flucarbazone application on the proportion of nodules successfully colonized by either of the inoculum strains R_1 or R_2. Nodules collected from field pea plants grown in a field treated with three rates of flucarbazone (0, 20, and 30 g a.i. ha^{-1}) 1 yr prior to field pea seeding.

	Goodale, SK			Beaverlodge, AB		
	0	20	30	0	20	30
	----------g a.i. ha^{-1}----------			----------g a.i. ha^{-1}----------		
Crown Average	1/6	2/6	3/6	4/6	4/6	4/6
Distal Average	2/6	1/6	2/6	4/6	4/6	4/6

* At each field site, four replicated plots were present for each treatment. From the plant samples collected to represent each replicated plot, six nodules were analyzed for each region (crown and distal), and the presence of the inoculum strain was recorded in terms of a fraction as in "X/6" where X signified the number of nodules containing an inoculum strain.

** The fractional values from the four replicated plots were averaged, rounded to the nearest whole number, and presented in the table.

Additionally, there were no statistically significant differences in the nodulation success by the inoculum strains when sampling regions were compared within each herbicide application rate (crown vs. distal) for Goodale (0 g a.i. ha^{-1}: $\chi^2 = 1.06$, $P = 0.30$; 20 g a.i. ha^{-1}: $\chi^2 = 2.02$, $P = 0.16$; and 30 g a.i. ha^{-1}: $\chi^2 = 0.87$, $P = 0.35$) and Beaverlodge (0 g a.i. ha^{-1}: $\chi^2 = 0.09$, $P = 0.76$; 20 g a.i. ha^{-1}: $\chi^2 = 0.78$, $P = 0.38$; and 30 g a.i. ha^{-1}: $\chi^2 = 0.00$, $P = 1.00$).

To reveal the potential difference between the inoculum rhizobial strains R_1 and R_2 in their infectiveness and susceptibility to flucarbazone, the information in Table 4.3 was re-arranged and presented in Table 4.4 so that each inoculum strain was shown separately. To maintain the clarity in data presentation, both sampling regions (crown and distal) of each replicate were combined before calculating the average values to be presented in Table 4.4. The combination of sampling regions changed the way in which the proportion of successful nodule colonization was presented from "X/6" to "X/12" where X still signified the number of nodules containing an inoculum strain.

When the proportions of successful colonization by the inoculum rhizobia were compared separately for each inoculum strain (R_1 or R_2) across the three treatments, statistically detectable differences were not found for Goodale (R_1: $\chi^2 = 2.34$, $P = 0.31$; R_2: $\chi^2 = 3.13$, $P = 0.21$) or Beaverlodge (R_1: $\chi^2 = 0.39$, $P = 0.82$; R_2: $\chi^2 = 3.34$, $P = 0.85$). Thus the use of flucarbazone was found to have no influence on the proportion of successful nodulation by inoculum rhizobia across the three treatments, regardless of the way in which the data were grouped (i.e., strains R_1 and R_2 together or separately).

In contrast, when the proportions of successful colonization by each inoculum strain were compared against each other for a given herbicide application rate to reveal the difference in their infectiveness (R_1 vs. R_2), some statistically significant differences were found.

Table 4.4 Influence of flucarbazone application on the proportion of nodules successfully colonized by the inoculum strains R_1 or R_2. Nodules collected from field pea plants grown in a field treated with three rates of flucarbazone (0, 20, and 30 g a.i. ha^{-1}) 1 yr prior to field pea seeding.

	Goodale, SK			Beaverlodge, AB		
	0	20	30	0	20	30
	----------g a.i. ha^{-1}----------			----------g a.i. ha^{-1}----------		
Strain R_1	1/12	2/12	2/12	6/12	6/12	7/12
Strain R_2	2/12	1/12	2/12	2/12	2/12	2/12

* At each field site, four replicated plots were present for each treatment. From the plant samples collected to represent each replicated plot, twelve nodules (i.e. six crown and six distal) were analyzed, and the presence of the inoculum strain was recorded in terms of a fraction as in "X/12" where X signified the number of nodules containing an inoculum strain.

** The fractional values from the four replicated plots were averaged, rounded to the nearest whole number, and presented in the table.

At Goodale, the inoculum strains R_1 and R_2 had comparable infectiveness under the treatments 0 g a.i. ha^{-1} ($\chi^2 = 0.92$, $P = 0.34$) and 30 g a.i. ha^{-1} ($\chi^2 = 0.71$, $P = 0.40$); however, the proportion of nodules colonized by strain R_1 was significantly greater than that of strain R_2 ($\chi^2 = 4.02$, $P < 0.05$) for the plants grown on a plot that received 20 g a.i. ha^{-1} of flucarbazone in year 1. At Beaverlodge, the proportion of nodules colonized by strain R_1 was significantly greater than that of strain R_2 for all three treatments (0 g a.i. ha^{-1}: $\chi^2 = 13.77$, $P < 0.05$; 20 g a.i. ha^{-1}: $\chi^2 = 14.28$, $P < 0.05$; and 30 g a.i. ha^{-1}: $\chi^2 = 14.76$, $P < 0.05$). Although such comparison of infectiveness between the inoculum strains was not the focus of this study, the data suggests the greater infectiveness of strain R_1 than strain R_2 at Goodale and Beaverlodge.

4.3.4 Verification of the nodule occupancy analysis

The sequencing results confirmed that the PCR-IGS products recovered from visually indistinguishable bands (i.e., bands with comparable migratory distances) were identical in the IGS gene sequences, except for one instance. A photograph contrasting the migratory distances of the sequenced samples from each pattern (A, B, C, R'_1, R'_2, R_1, and R_2) is presented in Fig. 4.4. Only the pattern R'_2 shows all of its replicate samples in Fig. 4.4 (i.e., $R'_{2\text{-}1}$, $R'_{2\text{-}2}$, and $R'_{2\text{-}3}$). Also, the sequencing results were compared against the microbial nucleotide sequence database (i.e., BLAST search of GenBank) to determine the most closely related strain (Table 4.5). The results from the BLAST search were consistent among the replicated sequence results for all of the patterns A, B, C, R'_1, R'_2, R_1, and R_2. Only the pattern R'_2 shows all of its replicate samples in Table 4.4 (i.e., $R'_{2\text{-}1}$, $R'_{2\text{-}2}$, and $R'_{2\text{-}3}$).

BLAST results indicated that the patterns A, B, and C, each of which with distinguishable mobility (Fig. 4.4), were 16S-23S rDNA IGS sequences of *Rhizobium leguminosarum* bv. *viceae* strains P233 and VD10B, and *Rhizobium* sp. CCBAU 83389, respectively. There was no entry available in the database that would match the sequence of band A to yield a minimum of 97% similarity, which is the minimum requirement for identity as suggested by Stackebrandt and Goebel (1994). The BLAST search of the pattern R'_1 and the reference strain R_1 indicated that both bands were 16S-23S rDNA IGS sequences of *Rhizobium leguminosarum* bv. *viceae* strain USDA2370, which confirmed the accuracy of the visual classification since the migration pattern R'_1 represents the sample nodule occupants with indistinguishable band mobility as the reference inoculum strain R_1.

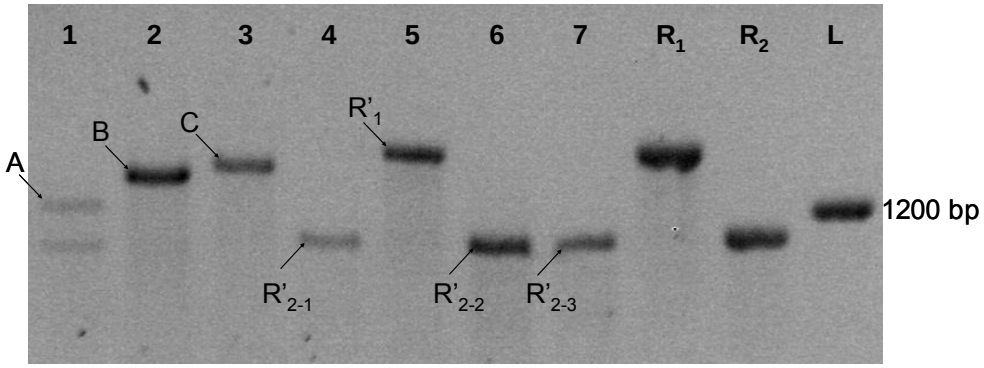

Figure 4.4 Banding patterns of PCR amplified IGS products ran on 2% agarose gel. Lane 1 to 7: *Rhizobium* strains extracted from nodule samples; Lane R_1 and R_2: Reference inoculum strains; Lane L: Low DNA Mass Ladder.

Table 4.5 Similarity comparison of sequences recovered from agarose gel bands against NCBI (National Centre for Biotechnology Information) GenBank database by using the online standard BLAST (Basic Local Alignment Search Tool).

Sequence designation[†]	Most related strain from GenBank (% sequence similarity by BLAST)[‡]	GenBank accession no. for most related sequences
A	*Rhizobium leguminosarum* bv. *viceae* strain P233 (93%)[§]	AY491951.1
B	*Rhizobium leguminosarum* bv. *viceae* strain VD10B (99%)	AY491949.1
C	*Rhizobium* sp. CCBAU 83389 (100%)	EF549553.1
R'_1	*Rhizobium leguminosarum* bv. *viceae* strain USDA2370 (99%)	AY491944.1
R_1	*Rhizobium leguminosarum* bv. *viceae* strain USDA2370 (100%)	AY491944.1
R'_{2-1}	*Rhizobium* sp. CCBAU 83268 (98%)	EF549538.1
R'_{2-2}	*Rhizobium* sp. CCBAU 83268 (99%)	EF549538.1
R'_{2-3}	*Rhizobium* sp. CCBAU 83268 (100%)	EF549538.1
R_2	*Rhizobium* sp. Trr-4 (98%)	AF510892.1

[†] Sequence designation are as labeled in Fig. 4.4. Bands A to C were similar to (but visually distinguishable from) one of the reference inoculum strains R_1 or R_2, while the bands R'_1 and R'_2 visually indistinguishable from the reference strains R_1 and R_2, respectively.
[‡] 97% sequence similarity is the minimum requirement for identity (Stackebrandt and Goebel, 1994).
[§] There was no entry available in the database that would match the sequence of band A to yield a minimum of 97% similarity.

With the reference inoculum strain R_1, the visual classification appeared to work consistently since all three sequenced samples of matching mobility ($R'_{1\text{-}1}$, $R'_{1\text{-}2}$, and $R'_{1\text{-}3}$) were identified as the same rhizobial strain as the reference strain R_1 according to the BLAST search of their 16S-23S rDNA IGS sequences. The reproducibility of visual classification was demonstrated further by the replicated samples of $R'_{2\text{-}1}$, $R'_{2\text{-}2}$, and $R'_{2\text{-}3}$ with visually indistinguishable mobility, when their sequence results were all identified as the same rhizobial strain upon the BLAST search (*Rhizobium* sp. CCBAU 83268). Interestingly however, when the gene sequence result of the inoculum strain R_2 was analyzed for its most closely related strain, it was identified as *Rhizobium* sp. Trr-4, which was different from the identity of R'_2 despite the fact that bands of R'_2 and R_2 were indistinguishable upon visual classification. When the IGS gene sequences of R'_2 and R_2 were directly compared against each other using the Clustral X program (Thompson et al., 1997), they were identified as being 93% similar to each other. The implications of these findings on the nodule occupancy analysis are discussed in Section 4.4.3.

4.4 Discussion

4.4.1 Influence of flucarbazone use on field pea production and inoculation success

Given the weather and soil conditions present at Goodale and Beaverlodge during year 1 and 2, the use of flucarbazone in wheat up to the field recommended application rate of 30 g a.i. ha^{-1} ($\approx$ 20 µg kg^{-1}) had no significant influence on field pea production and nodulation as indicated by the absence of statistically detectable differences in: shoot and root weight, seed yields, nitrogenase activity (ARA), total nodule number and weight, and N concentration (µg mg^{-1}) and $\delta^{15}N$ (‰) of above-ground plant material. Because the growth chamber experiment (Chapter 3) demonstrated the significant reduction of plant growth and nodulation in the presence of

high enough concentrations of flucarbazone in soil, the results from the field experiment certainly point to the absence of soil residual flucarbazone one year after application, or the amount of flucarbazone carried over into the following season was too low to influence plant and nodule development.

Referring back to the hypothesis statement: *the presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia*, it is likely safe to assume that the presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia when the concentrations of soil residual flucarbazone is low, which was probably the case for the present field study. This assumption is based on the comparable inoculation success of field pea among the three treatments (0, 20, and 30 g a.i. ha^{-1} flucarbazone-treated plots) as indicated by the lack of statistically detectable differences in plant growth, grain yields, nodulation, nitrogenase activities, and plant tissue N content among the three treatments. In other words, the lack of herbicidal influence on field pea production, nodulation, and N_2-fixation point to the fact that the level of residual flucarbazone present (if any) in this field study did not interrupt the inoculum rhizobia from accomplishing their task of establishing an effective symbiotic relationship with the host plant. The presence of soil residual flucarbazone at the field sites were tested and the results are reported in Chapter 5.

Having speculated the lack of residual herbicidal impact on inoculation success as above, a possibility was recognized that the proportion of nodules occupied by the inoculum rhizobial strains may differ among the three treatments without affecting any of the parameters measured for the present study. Thus, an attempt was made to genetically identify the origin of nodule occupants using a molecular microbiological method, and the findings are presented in Sections 4.4.3 and 4.4.4.

As discussed in Chapter 3, some published data was available on the influence of non-aged AHAS inhibitors directly applied to leguminous plants and/or rhizobial

bacteria. However, published data dealing with the influence of 'residual' AHAS inhibitors on legume production was very rare at the time of writing. Moyer et al. (1990) conducted a field experiment to study the influence of residual AHAS-inhibiting herbicide (chlorsulfuron) on rotational crops by applying the herbicide in wheat one year, then growing various crops on the same plot the following season. They reported that when the recommended field rate of chlorsulfuron was used in wheat, the subsequent field pea yield was reduced by approximately 75%. Moyer et al. (1990) attributed the cause of the severe rotational crop injury to the high pH (pH 8) and low SOM (2%) of the field soil, along with other climatic conditions that favoured the likelihood of herbicide carryover. The influence of residual chlorsulfuron on shoot and root dry biomass, nitrogenase activity, nodule formation, plant tissue N and $\delta^{15}N$ contents were not reported by Moyer et al. (1990). The findings of Moyer et al. (1990) support the potential of AHAS inhibitors to cause injury in rotational crops. However, since the likelihood of herbicide carryover is largely influenced by the soil and environmental conditions and the nature of the herbicide itself, the risk of the rotational crop injury induced by residual AHAS inhibitors needs to be assessed on a case-by-case basis.

4.4.2 Influence of location and climate on field pea production and inoculation success

Although the comparison of plant growth parameters between field sites was not the focus of the present field experiment, there was a sharp contrast in plant and nodule development which was noteworthy. In general, the Beaverlodge site was less productive than the Goodale site. For example, field pea grown at Beaverlodge had 30% less shoot biomass (Fig. 4.1a and 4.2a), 35% less seed yield (Fig. 4.1c and 4.2c), and 82% fewer nodules (Fig. 4.1e and 4.2e) than pea grown at Goodale.

It has been reported that nodules have a strong demand for C, and can grow at the expense of root growth in both the vegetative and flowering stages (Voisin et al.,

2003). This may explain the presence of greater root biomass on control plants at Beaverlodge (with fewer nodules) than that of Goodale (Fig. 4.1b and 4.2b). Despite the presence of greater root biomass however, the control plants of Beaverlodge had less N accumulation (%) in the shoot than that of Goodale (Fig. 4.1g and 4.2g).

One of the reasons for the considerable difference in field pea development between the two locations might have been the difference in precipitation where Goodale received above the 30-yr long term average from May to September for both years 1 and 2 (78 and 54% above the 30-yr average, respectively), while Beaverlodge received less precipitation than the 30-yr average during the same time period for both years 1 and 2 (20 and 46% below the 30-yr average, respectively) (Table 4.2). Aside from its obvious influence on plant development, soil moisture availability has important implications for the present study because soil moisture content is one of the key factors influencing the rate of herbicide degradation in soil (Eliason, 2003). Thus, although none of the plant growth parameters suggested the presence of residual flucarbazone in soil, it is possible that the precipitation difference at these sites caused a difference in the way that flucarbazone was degraded at each location. Further analysis on the residual flucarbazone was conducted on the soil samples from both field sites, and this information is presented in Chapter 5.

4.4.3 Verification of the nodule occupancy analysis

As described previously, the genetic identification of sample nodule occupants in this study (inoculum vs. native) was conducted using the PCR-IGS. By visually comparing the PCR-IGS products of the reference inoculum rhizobia and sample nodule occupants based on their migratory distance upon agarose gel electrophoresis (Section 3.2.4.2), nodule samples were classified as either having ‘matching’ or ‘non-matching’ bands as the reference strains. Those nodules with indistinguishable band mobility as the

reference strains were then assumed to have been colonized by the inoculum rhizobia. This method was used in both the growth chamber study (Chapter 3) and the field experiment (Chapter 4) to examine the influence of flucarbazone on successful nodulation initiated by inoculum rhizobial strains.

The sequencing results of PCR-IGS products (Section 4.3.4) clearly demonstrated the reproducibility of the visual classification method because the gene sequences of the replicated samples for each migration pattern (i.e., A, B, C, R'_1, R'_2, R_1, and R_2) were consistently identified to be the same rhizobial strain according to the BLAST search (Section 4.3.4). The sequencing results also demonstrated that those sample nodule occupants visually classified as having the migration pattern R'_1 (and thus assumed to be the inoculum rhizobial strain R_1) were indeed the inoculum strain R_1. These findings supported the usefulness and accuracy of the visual identification method in recognizing sample nodules containing the inoculum rhizobial strain R_1. Since the growth chamber experiment (Chapter 3) was conducted with the inoculum strain R_1 only, the results of genetic analysis on nodule occupants, as presented in Section 3.3.4, is reliable for the strain tested.

In contrast, the visual identification method was not effective in accurately recognizing sample nodules containing the inoculum rhizobial strain R_2. While the BLAST search identified the gene sequence of the inoculum strain R_2 as *Rhizobium* sp. Trr-4, those sample nodule occupants with indistinguishable band mobility as the inoculum strain R_2 were identified as *Rhizobium* sp. CCBAU 83268 according to the BLAST search (Section 4.3.4). In other words, the visual identification method was not able to distinguish a nodule occupant of non-inoculum origin (*Rhizobium* sp. CCBAU 83268) from the inoculum rhizobial strain R_2 (*Rhizobium* sp. Trr-4).

As reviewed in Section 2.5.2.1, the usefulness of the IGS regions primarily stems from its variability in sequence and length. To fully exploit the dual variability of

IGS, the use of methods such as restriction fragment length polymorphism (RFLP) or denaturing gradient gel electrophoresis (DGGE) would be ideal (Section 2.5.2.2). However, these methods necessitate extra processing and prolonged preparation, and thus were not suitable for the present study that involved a large number of nodule samples for genetic identification. Therefore, the PCR-IGS products were analyzed using a simple and rapid method of agarose gel electrophoresis that differentiates PCR-IGS products exclusively on the basis of their length variation.

In the present study, the lengths of the PCR-IGS products obtained from inoculum and native rhizobia varied from approximately 600 to 1400 bp long (Section 4.3.3). Such length variation was, in fact, sufficient to correctly differentiate most of the rhizobial strains encountered in this experiment, including those strains with similar IGS length (i.e., migration patterns A, B, C, R'_1, and R'_2). Unfortunately however, the sequencing results revealed the presence of a native rhizobial strain (*Rhizobium* sp. CCBAU 83268; migration pattern R'_2) whose IGS region is very similar in length to that of the inoculum strain R_2. This meant that it was not possible to distinguish *Rhizobium* sp. CCBAU 83268 from the inoculum strain R_2 based solely on the PCR-IGS length which, in turn, meant that the visual identification method used for the present study has the potential to falsely identify the nodule occupant of non-inoculum origin (*Rhizobium* sp. CCBAU 83268) as the inoculum rhizobial strain R_2.

4.4.4 Influence of flucarbazone on nodulation success by an inoculum strain

The genetic analysis of nodule occupants conducted on the nodule samples collected from the current field experiment revealed that the three rates of flucarbazone (0, 20, and 30 g a.i. ha^{-1}) applied in year 1 had no influence on the nodulation success by the inoculum rhizobial strains in year 2, whether both strains R_1 and R_2 were analyzed collectively or separately (Tables 4.3 and 4.4).

Although the sequencing results revealed the potential overestimation on the nodulation success of strain R_2, the visual classification of strain R_1 appears accurate, and it was consistently found by the growth chamber study (Chapter 3) and the present field experiment that the nodulation success of strain R_1 is unaffected by the use of flucarbazone. Therefore it appears safe to conclude that the use of flucarbazone up to the field recommended application rate does not influence the nodulation success by the inoculum rhizobial strains, if the soil and environmental conditions are comparable to those observed during the present field study.

As discussed in Chapter 3, for a given herbicide to selectively influence the nodulation success by an inoculum strain, the herbicide would have to discriminatorily hinder the survival, chemical signalling, and/or nodulation ability of the inoculum strain while native strains of rhizobia continue to nodulate the host plant. Given the lack of relevant published data, it was not possible to verify if flucarbazone would have such selectivity to cause differences in the proportion of successful nodulation by inoculum rhizobia. However, the results of the growth chamber experiment (Chapter 3) showed that the flucarbazone addition at high enough concentrations reduced the nodule count without influencing the nodulation success by an inoculum strain. These results seem to indicate that the influence of flucarbazone on nodule occupancy is generally applicable to all rhizobial strains (both inoculum and native), rather than being discriminatory against the inoculum strains tested in this study.

4.5 Conclusion

The results from this experiment suggest that the use of flucarbazone in wheat, up to the field recommended application rate of 30 g a.i. ha^{-1} (≈ 20 μg kg^{-1}) does not influence the production and nodulation success of field pea in the subsequent cropping season, if the weather and soil conditions favour degradation and/or dissipation of the

herbicide, as described in the present experiment. Results of a growth chamber experiment (Chapter 3) indicated that the presence of flucarbazone in soil, at high enough concentrations, has the potential to hinder pea growth and nodulation. Thus, the reason for the absence of detectable effects of residual herbicide in the present field experiment may be that both field sites did not have any flucarbazone remaining in soil one year after application, or the amount of flucarbazone carried over into year 2 was low enough not to influence plant and nodule development. Further analysis on the presence of soil residual flucarbazone was conducted for both sites, and results are presented in Chapter 5.

It can be speculated that if the field sites from the present study had the combination of soil and environmental conditions that are conducive to herbicide persistence in soil, the growth parameters observed in the present field study might have shown signs of rotational injury. To elucidate the influence of soil and environmental conditions on rotational crop injury caused by residual flucarbazone, it is necessary to gather more data by repeating the field experiments under various soil and climatic conditions.

5 RESIDUAL FLUCARBAZONE CONCENTRATIONS AND IMPACT ON MICROBIAL POPULATIONS IN FIELD INCUBATED SOILS

5.1 Introduction

Adequate weed control is vital in sustaining high crop yields, and consequently herbicides have become a key component in modern agricultural production. Ideally, herbicides control weeds during the growing season of the treated crop and dissipate to non-toxic levels before the next crop is seeded; however, some herbicides may persist longer than desired and injure or kill subsequently planted crops (Hanson et al., 2004). Such persistence of residual herbicides in the soil is a disadvantage for producers who aim to maximize crop diversity and productivity through rotation because it limits the flexibility of crop rotation planning (Beckie and McKercher, 1989).

When herbicides are applied, most of the spray solution contacts the soil and it may affect soil microorganisms that are important for sustainable agriculture, e.g., recycling of plant nutrients, maintenance of soil structure, and symbiotic assistance of crop growth (Vieira et al., 2007). The herbicidal impact on soil microbes can occur instantly at the time of application and/or have long-lasting effects into the following cropping years.

The potential negative impact of residual herbicide on soil microbial populations is a serious concern in western Canada where wheat production is often followed with annual grain legumes such as field pea (*Pisum sativum* L.), chickpea (*Cicer arietium* L.), and lentil (*Lens esculenta* L.) in a sequential rotation. The basis for the concern is the potential interference with the symbiotic association between the host legume and the

Rhizobium bacteria. Given its sensitivity to changes in many environmental factors and soil conditions, the symbiotic association between the host legume and rhizobia may be affected by the presence of agrochemical residues which in turn, may negatively influence inoculation success, N_2 fixation, and yield of grain legumes.

Amongst many herbicides used in wheat production, acetohydroxyacid synthase (AHAS)-inhibiting herbicides warrant special attention given their residual properties (Colborn and Short, 1999), acute plant toxicity at low concentrations in soil (Santel et al., 1999), and their ability to influence both host plant and the bacterial symbiotic partner (Duggleby and Pang, 2000). In fact, the reduction in plant/nodule production and N_2 fixation induced by various AHAS-inhibiting herbicides in different leguminous plants has been reported (Gonzalez et al., 1996; Royuela et al., 2000; Anderson et al., 2004; Zawoznik and Tomaro, 2005).

The present thesis project focused on one AHAS inhibiting herbicide (flucarbazone-sodium), and studied its potential to persist into the subsequent cropping season to influence inoculation success during field pea production. A growth chamber experiment (Chapter 3) clearly demonstrated the susceptibility of field pea to the presence of flucarbazone in soil where it was shown that the herbicide has the potential to hinder the proper development of the plant and root nodules when high enough concentrations of flucarbazone were present in the soil (5 μg kg^{-1}; approximately 25% of recommended field application rate). Despite such findings from the growth chamber study, the use of flucarbazone in wheat, up to the field recommended application rate of 30 g a.i. ha^{-1} (≈ 20 μg kg^{-1}), did not influence the production and nodulation success of field pea in the subsequent cropping season during our field experiment (Chapter 4).

In an attempt to explain the lack of herbicidal influence in the field experiment, further analyses were conducted on the field soil samples to determine the presence/absence of soil residual flucarbazone. Also, the native rhizobial population and

total soil microbial population in the flucarbazone-treated soil samples were determined to find out if the use of flucarbazone in year 1 had caused a lasting negative influence on the soil microbial population size that is detectable in year 2.

5.2 Materials and Methods

5.2.1 Description of field sites and soil characteristics

Detailed descriptions of field sites as well as the soil characteristics are provided under Section 4.2.1. Briefly, field experiments were conducted in the spring/summer of 2005 and 2006 at the field sites in Saskatchewan and Alberta. In order to simulate flucarbazone carryover during crop rotation, wheat was grown in 2005 (year 1), and was treated with three rates of flucarbazone-sodium (0, 20, 30 g a.i. ha^{-1}) to control weeds in wheat, where the recommended field rate is 30 g a.i. ha^{-1} (≈ 20 µg kg^{-1}). The control plots that received no flucarbazone were treated with non-residual herbicides containing no AHAS inhibitor (i.e., bromoxynil and clodinafop mix) at the field recommended application rates to control weeds. At each site, all treatments were replicated four times, and arranged in a randomized complete block design. Prior to seeding pea in 2006 (year 2), soil samples (0- to 10-cm depth) were collected from each replicated plot with a shovel, and bulked together to obtain a composite sample for each treatment at each location. At each replicated plot, three to five sampling locations were randomly selected, avoiding the perimeters of the plot. As soon as the soil samples were returned to the laboratory, a portion of each composite soil sample was ground to pass through a 2-mm sieve, and stored in a freezer (- 20 °C) for microbial analyses. The rest of the composite soil samples were ground to pass through a 2-mm sieve, and stored in a walk-in refrigerator (5 °C) to be used for mustard bioassay and chemical analysis.

5.2.2 Measurement of residual flucarbazone concentrations in experimental plot soils

5.2.2.1 Mustard bioassay

A mustard bioassay was conducted by using oriental mustard seeds (*Brassica juncea* L. var. Cutlass) as described in Szmigielski et al. (2008) to determine the bioavailable concentrations of residual flucarbazone at the experimental sites one year after herbicide application. Firstly, standard bioassay curves for each site were constructed by using soil sample from the control plots with no flucarbazone residues. The standard curves were needed to relate the bioassay results of the herbicide-treated soils to the soil residual flucarbazone concentrations. Fifty grams of the sieved control soil samples were spiked with various concentrations of flucarbazone solutions together with an appropriate amount of water in order to obtain subsamples containing final flucarbazone concentrations of 0, 1, 3, 5, 10, and 20 μg kg^{-1} at 100% field capacity (FC). The 20 μg kg^{-1} level is approximately equivalent to the recommended rate of field application (30 g a.i. ha^{-1}) assuming that flucarbazone remains in the top 10 cm layer of soil that has a bulk density of 1.3 g cm^{-3} (Eliason et al., 2004). The spiked subsamples were thoroughly mixed, transferred to Whirl-Pak™ bags (6 cm wide x 10 cm long), and gently formed into a layer which was approximately 6 cm wide, 8 cm long, and 1 cm thick. Six oriental mustard seeds were planted in each bag at a depth of approximately 2 mm, and the soil surface was covered with a 0.5 cm layer of white plastic beads to minimize soil moisture loss. Plants were grown under fluorescent light (12 μmol m^{-2} s^{-1} photon flux density; 24 h light) for 3 d at room temperature. Bags were watered every day to 100% FC by adding distilled water up to a predetermined weight until harvest, upon which the root length was measured using a ruler. Each herbicide concentration was replicated four times (i.e., 4 bags x 6 plants/bag = 24 plants per treatment). Following the root length measurement, the inverse relationship between the increasing

herbicide concentration and the decreasing average root length of the spiked treatments was plotted against each other. Upon plotting, the average root length of flucarbazone-spiked plants was expressed in terms of percentage values relative to the root length of control, which is set to 100%. More specifically, this was done by using the formula: $(L_t/L_0) \times 100$, where L_t is the average root length measured in the flucarbazone-spiked soil and L_0 is the average root length in the untreated soil (Eliason et al., 2004). A sigmoidal curve was then fitted using the following mathematical formula (Seefeldt et al., 1995):

$$y = C + \frac{D - C}{1 + (x/I_{50})^b} \qquad (5.1)$$

where *D* corresponds to the mean root growth length of the control plants. When the curve is plotted, *D* is set to 100% which corresponds to the mean root growth length when the roots are allowed uninhibited growth, and thus, also signifies the upper limit of the sigmoidal curve. The value *C* corresponds to the mean root growth length of the control plants at very high doses of root growth inhibitor (i.e., flucarbazone). The value *C* is extrapolated from the sigmoidal curve that best fits the plotted data points obtained from the above mentioned bioassay. Moreover, *C* corresponds to the flattened portion at the bottom of the sigmoidal curve, and it also represents the lower limit of the curve. Note that the lower limit is not necessarily zero in biological responses (Seefeldt et al., 1995). The growth inhibitor concentration ($\mu g\ kg^{-1}$ soil) that caused the average root growth length of the treated plants to fall midway between *D* (upper limit) and *C* (lower limit) is then referred to as I_{50}. Since the lower limit (*C*) is not always zero, I_{50} does not necessarily correspond with the 50% point on the Y-axis. Finally, *b* in the above formula represents the slope of the curve at the I_{50} value. Given the definitions of *C* and *D*, the

model was fitted with constraints $C \geq 0$ and $D \leq 100$. Estimated values for C, I_{50}, and b are given in Table 5.1., and the standard curves for both locations are presented in Fig. 5.1.

After obtaining the standard curves, the mustard bioassay was conducted using the field soil samples collected from plots that were sprayed with flucarbazone one year prior to the sample collection at three different rates (0, 20, and 30 g a.i. ha^{-1}). Upon harvest, root length was measured, averaged, and the plant response to the soil samples was related to residual herbicide concentration in soil using the standard curves.

5.2.2.2 Chemical analysis

Along with the bioassay, the same set of flucarbazone-treated soil samples were tested chemically for the presence of residual herbicide. The chemical extraction analysis of the soil samples was conducted by ALS Laboratory Group (Edmonton, AB), which used the modified EPA test method to determine the concentration of soil residual flucarbazone and its degradation products (i.e., metabolites). Detailed procedures are described in EPA (2007) online test method collections. Briefly, in order to extract flucarbazone and its metabolites, acetonitrile was added to a soil sample and shaken in a flask. The extract was centrifuged and the supernatant was cleaned by elution through an acetonitrile pre-washed C18 cartridge. The extractant was then analyzed for the concentration of flucarbazone and its degradation products with high-performance liquid chromatography.

Table 5.1 Estimated parameters for the root growth inhibition bioassay standard curves for Goodale, SK and Beaverlodge, AB.

Soil (location)	C† ± SE	D‡	I_{50}§ ± SE	b¶ ± SE
Goodale	19.1 ± 4.4	100	2.7 ± 0.3	- 4.5 ± 2.8
Beaverlodge	24.1 ± 9.7	100	8.8 ± 1.2	- 3.4 ± 1.4

† C = lower limit of the sigmoidal curve (%).

‡ D = upper limit of the sigmoidal curve (%). 100% by definition.

§ I_{50} = concentration corresponding to 50% inhibition (µg kg^{-1}).

¶ b = slope of the curve around I_{50} value.

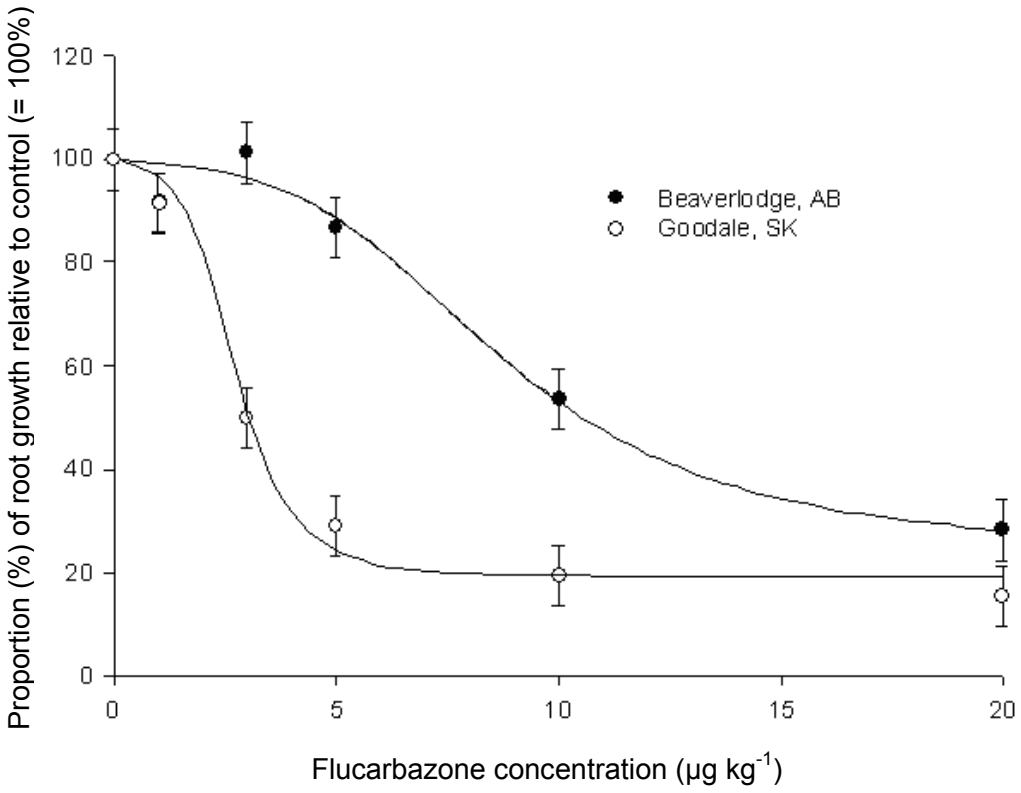

Figure 5.1 The root growth inhibition bioassay standard curves for Goodale and Beaverlodge. Each data point represents mean ± standard error. Root length of control plant (0 µg kg^{-1}) was set to 100%, and the root length of the treated plants were expressed in terms of a proportion (%) relative to the root length of control (= 100%).

5.2.3 The effect of flucarbazone application on the native rhizobial population

5.2.3.1 Plant infection most probable number of native rhizobial population

The population size of the native strains of *R. leguminosarum* bv. *viceae* in the field soil samples collected prior to field pea seeding in year 2 (2006) from Goodale and Beaverlodge was determined using the plant infection count, also known as the most-probable-number method (MPN) (Weaver and Frederick, 1972). This method uses a host leguminous plant to 'trap' compatible rhizobial strains and thereby enumerate the number of viable and infective rhizobia in a given soil sample in the presence of other microorganisms. For the present experiment, 10 g of sieved composite soil samples from each treatment (0, 20, and 30 g a.i. ha^{-1} of flucarbazone applied in year 1) were successively diluted in glass bottles containing 95 mL of sterile phosphate buffer solution (PBS) over the dilution range of 10^{-1} to 10^{-6}. Surface sterilized and pre-germinated field pea seeds (*Pisum sativum* cv CDC Mozart) were then inoculated with each dilution at 1 mL per seed, and grown in a growth pouch (Mega International, West St. Paul, MN) containing sterile Fahraeus N-free nutrient solution (1957). The growth pouches were kept in a growth chamber with a 16 h photoperiod and day/night temperatures of 22/18 ºC, during which time the development of the plants and nodules were observed daily, and the N-free nutrient solution was replenished as required. Nodulation was evident after 2 wk, and the final observation was made after 4 wk to record presence or absence of root nodules on the four replicated plants for each dilution. The MPN of the native *R. leguminosarum* bv. *viceae* strains present in a gram of field soil sample was then computed according to Somasegaran and Hoben (1994) using the appropriate statistical tables for the dilution and number of replications for the present study.

5.2.3.2 Colony forming unit plate count of total viable soil microbial population

Above mentioned serially-diluted soil solutions prepared for the MPN assessment were used to enumerate total viable soil microbial population by the plate count of colony forming units (CFU). Detailed procedures are described by Page (1982). Briefly, for each dilution of a given soil sample (10^{-1} to 10^{-6}), 0.1 mL of soil solution was evenly spread on a 1/10 strength tryptic soy agar (1/10 TSA) plate in quadruplicate and incubated at room temperature. After 3 d of incubation, the dilution that yielded from 30 to 300 colonies per plate was selected and colonies were counted to compute the number of CFU per one gram of field sample soils. The CFU count of each soil sample was conducted twice to ensure the accuracy of the results.

5.3 Results

5.3.1 Mustard bioassay

The results from the mustard bioassay and chemical extraction are summarized in Table 5.2. Bioassay results for both field sites (Goodale and Beaverlodge) indicated that there were no statistically detectable differences (ANOVA; $P < 0.05$) in the root length of oriental mustard grown in soil samples representing three treatments (i.e., application of 0, 20, and 30 g a.i. ha^{-1} of flucarbazone in year 1 in wheat), thus it was not necessary to utilize the estimated values obtained from the standard curves (i.e., C, I_{50} ,and b in Table 5.1). The lack of root growth inhibition in this experiment indicates that the mustard bioassay did not detect any residual flucarbazone in the treated plots at both field sites in year 2.

Although, the comparison of standard curves between field sites was not the focus of the present experiment, the difference was noted where considerably more root length inhibition occurred in Goodale soils relative to Beaverlodge for a given flucarbazone spiking concentration (Fig. 5.1). In fact, it only took an average of 2.6 µg

kg^{-1} of flucarbazone to cause 50% root length inhibition in Goodale soil, whereas the Beaverlodge soil required over three times the concentration (8.9 μg kg^{-1} on average) of flucarbazone to have the same root length inhibition (Table 5.1).

Table 5.2 Concentration of soil residual flucarbazone one year after application detected by mustard bioassay and chemical extraction.

	Mustard Bioassay		Chemically extracted residual flucarbazone (μg kg^{-1})
	Root length ± SE (cm)	Detection amounts (μg kg^{-1})	
Goodale:			
Control	8.1 ± 0.1	0†	n.a.
20 g a.i. ha^{-1}	8.6 ± 0.6	Undetectable	0.4
30 g a.i. ha^{-1}	8.6 ± 0.3	Undetectable	0.5
Beaverlodge:			
Control	8.4 ± 0.6	0†	n.a.
20 g a.i. ha^{-1}	8.4 ± 0.4	Undetectable	BDL‡
30 g a.i. ha^{-1}	8.8 ± 0.3	Undetectable	BDL‡

† Zero by definition.

‡ BDL denotes Below Detection Limit. Flucarbazone-sodium was not present at concentrations greater than the detection limit of 0.1 μg kg^{-1}. However, the flucarbazone degradation product, sulphonamide, was detected in Beaverlodge soil samples at 2.3 and 4.0 μg kg^{-1} for 20 g and 30 g a.i. ha^{-1} soil, respectively.

5.3.2 Chemical extraction analysis

The chemical extraction detected residual flucarbazone and its metabolite in the flucarbazone treated soil samples collected from Goodale and Beaverlodge (Table 5.2). The concentration of residual flucarbazone at Goodale was found to be 0.4 and 0.5 µg kg^{-1} for the 20 and 30 g a.i. ha^{-1} flucarbazone treated plots, respectively. At Beaverlodge, flucarbazone residue in soil was found to be below the detection limit of 0.1 µg kg^{-1} for both treatments (20 and 30 g a.i. ha^{-1}).

Degradation products of flucarbazone were not found in Goodale soil samples of both treatments at concentrations greater than the detection limit. In contrast, one of the flucarbazone degradation products, sulphonamide, was present in soils of Beaverlodge at 2.3 and 4.0 µg kg^{-1} for the 20 and 30 g a.i. ha^{-1} flucarbazone treated plots, respectively.

5.3.3 Plant infection most probable number of native rhizobial population and colony forming unit plate count of total viable soil microbial population

There were no statistically detectable differences (ANOVA; $P < 0.05$) in the results of plant infection MPN and total microbial CFU count when each experiment was conducted on the soil samples representing all of the treatments (0, 20, and 30 g a.i. ha^{-1} of flucarbazone applied in year 1) of both Goodale and Beaverlodge sites (Table 5.3). Relatively low total microbial populations at Goodale and Beaverlodge may reflect the impact of dry soil conditions at the time of soil sampling in late April 2006 at these locations.

Table 5.3 Native rhizobial and total microbial population one year after flucarbazone application approximated by plant infection MPN and total microbial CFU count.

	Native pea rhizobial population (per g soil)	Total microbial population (per g soil)
Goodale:		
Control	1.0×10^3	1.6×10^6
20 g a.i. ha^{-1}	5.8×10^2	1.5×10^6
30 g a.i. ha^{-1}	5.8×10^2	1.5×10^6
Beaverlodge:		
Control	1.0×10^2	1.2×10^6
20 g a.i. ha^{-1}	1.0×10^2	1.2×10^6
30 g a.i. ha^{-1}	1.7×10^2	1.1×10^6

5.4 Discussion

5.4.1 Mustard bioassay and chemical extraction results, and their implications on field pea production

According to the chemical extraction results, the concentration of residual flucarbazone one year after application at Goodale was found to be 0.4 and 0.5 μg kg^{-1} for the 20 and 30 g a.i. ha^{-1} flucarbazone treated plots, respectively. In contrast, residual flucarbazone was not found in Beaverlodge soil samples at concentrations greater than the detection limit of 0.1 μg kg^{-1} for both treatments of 20 and 30 g a.i. ha^{-1}. Such concentrations of residual flucarbazone at both field sites were not high enough to cause any inhibitory effects on the root development of oriental mustard grown in these soils. These results were consistent with the findings of Szmigielski et al. (2008) who evaluated the use of mustard root-length bioassay in predicting crop injuries from soil residual flucarbazone, and reported that the detection limit of the bioassay to be approximately 1 μg kg^{-1}.

As reviewed in Section 2.2.3, plant species vary in their sensitivity to AHAS inhibitors. In an unpublished field experiment, Sapsford et al. (2006) compared the sensitivity of fifteen crops to residual flucarbazone in field plots in western Canada, eleven months after the initial application of the herbicide at various rates. They reported the presence of visually observable injury in two varieties of mustard (*Brassica juncea*) when they were grown on soils that received two-thirds of the recommended rates (20 g a.i. ha^{-1}) of flucarbazone in year 1. Field pea, on the other hand, had much greater tolerance than these mustard varieties where no injury was observed when grown on plots that received 40 g a.i. ha^{-1} of flucarbazone in year 1 (Sapsford et al., 2006). Sapsford et al. (2006) reported that visually observable injury was only present in field pea when it was grown on those plots that received double the recommended concentration (60 g a.i. ha^{-1}) of flucarbazone in year 1.

Given the lack of published data, it was not possible to directly compare the AHAS inhibitor tolerance among mustard varieties used in the present experiment and those tested by Sapsford et al. (2006). However, it is expected that any variety of mustard of the same species (i.e., *Brassica juncea*) would behave in an identical or very similar manner to the presence of residual herbicide. Therefore, it is safe to assume that field pea has much greater tolerance to the presence of soil residual flucarbazone than oriental mustard used in the present bioassay. Thus the results of the bioassay and chemical extraction support the results from our field experiment (Chapter 4) shows that the use of flucarbazone in wheat in year 1 (2005), up to the field recommended application rate of 30 g a.i. ha^{-1}, did not influence the production and nodulation success of field pea in the subsequent cropping season.

5.4.2 Influence of soil characteristics and precipitation on flucarbazone persistence

Aside from the lack of residual herbicidal influence on mustard root growth in soils from both locations, there were some other noteworthy observations. Firstly, the difference was noted during the standard curve construction where considerably more root length inhibition occurred in Goodale soils relative to Beaverlodge for a given rate of flucarbazone application (Fig. 5.1). Secondly, the concentrations of chemically extractable residual flucarbazone were greater in Goodale soils than Beaverlodge, while one of the metabolites (sulfonamide) was only detected at Beaverlodge (Table 5.2). Such differences did not influence the bioassay results and the analyses of such differences between locations was not the objective of this experiment. However, the factors causing such variations among locations can have practical and agronomical implications.

As reviewed in Section 2.2.2, sorption–desorption interactions of herbicides with soil determine the availability of the chemical in soil (Koskinen et al., 2006). It was reported that the bioavailability of herbicide in soil is strongly influenced by the soil

organic matter (SOM) content because soils with high SOM content have increased herbicide retention capacity due to the presence of a large number of adsorption sites (Loux et al., 1989). The SOM contents of Goodale and Beaverlodge were 2.2 and 3.9%, respectively, and the lesser concentration of SOM at Goodale may partly explain the existence of greater root length inhibition in Goodale soil relative to Beaverlodge soil when soils were spiked with herbicide. Though generally considered to have less influence on chemical adsorption than SOM, soil texture (relative composition of sand, silt, and clay) also play an important role in herbicide adsorption (Griffin, 2006) because clay provides a much greater soil surface area than sand for chemical adsorption (Calvert, 1980; Peter and Weber, 1985). The clay contents of Goodale and Beaverlodge were 23 and 51%, respectively. Together with the lesser concentration of SOM at Goodale, the lesser proportion of clay present in Goodale soil might have contributed to the greater root length inhibition in Goodale soil during standard curve construction, relative to Beaverlodge.

Regarding the differences in the concentrations of chemically extractable residual herbicide and metabolite at the two field sites, the interpretation of such locational variation was not straightforward due to the potential influence exerted by both soil and climatic conditions. As reported previously, the concentrations of chemically extractable residual flucarbazone were greater in Goodale soils than Beaverlodge, except for one of the metabolites (sulfonamide), which was only present in greater concentrations at Beaverlodge (Table 5.3). This may indicate that flucarbazone was degraded relatively slowly at Goodale such that residues were detected, one year after application, in the form of flucarbazone. Meanwhile, it is also possible that flucarbazone degradation proceeded much more rapidly at Beaverlodge so that flucarbazone and other further-degradable metabolites had dissipated, leaving

sulfonamide, which is resistant to aerobic metabolism in soil (EPA, 2000), to accumulate. The relevance of this hypothesis is examined and presented below.

As reviewed in Section 2.2.1, the primary route of degradation for flucarbazone is believed to be microbial (Santel et al., 1999). Generally, environmental factors such as adequate soil moisture and high SOM encourage microbial growth and reproduction (Griffin, 2006), which in turn provides an ideal environment for microbial degradation of herbicides. In support of such observations, Eliason et al. (2004) studied flucarbazone degradation in soils of western Canada and reported that flucarbazone dissipation occurred more rapidly in wetter soils with higher organic C content.

As described in Section 4.3.1, the precipitation data indicates that Goodale soil received more rainfall than Beaverlodge for both year 1 and 2 growing seasons which, contrary to our hypothesis, may have favoured degradation of flucarbazone at Goodale. However, periodic observations were not made to monitor the exact change in soil moisture availabilities during the course of field experiment. Given the differences in soil characteristics at the two locations, it was not possible to conclusively state that the greater rainfall led to greater soil moisture availability throughout the growing seasons.

As for the influence of soil properties on flucarbazone degradation, greater SOM content in Beaverlodge soil is in agreement with the above hypothesis. On the other hand, it has been reported that herbicide dissipation rates are generally slower in heavier textured soils because of increased adsorption (Walker, 1991). Since Beaverlodge had much greater clay content (51%) than Goodale (23%), the soil texture does not support the above hypothesis.

5.4.3 Plant infection most probable number of native rhizobial population and colony forming unit plate count of total viable soil microbial population

There were no statistically detectable differences in plant infection MPN and total microbial CFU counts when each experiment was conducted on the soil samples representing all of the treatments (0, 20, and 30 g a.i. ha^{-1} of flucarbazone applied in year 1) of both field sites. The lack of detectable differences among treatments indicated that the use of flucarbazone up to the field recommended application rate (30 g a.i. ha^{-1}) in the present experiment did not have a lasting negative influence on the total microbial population and the native rhizobial population, which can be detected a year after application. This does not imply that flucarbazone application did not have any influence in year 1 at the time of herbicide application in wheat when flucarbazone solution came in direct contact with the soil and its microbial inhabitants. Rather, the results from infection MPN and total microbial CFU count indicate that any influence of flucarbazone on soil microbial populations at the time of herbicide application did not persist into the subsequent cropping season given the soil and environmental conditions which were present during our field experiment.

To date, the existence of side effects on the soil bacterial population caused by the AHAS inhibitors or the variable sensitivity of bacteria to AHAS inhibitors have not been adequately investigated in the field. Also, our experiment did not examine the potential shift in microbial community structure caused by the herbicide application. Therefore, the results from the present experiment pointing to the lack of herbicidal influence on soil microbial population need to be interpreted with caution.

Finally, concerning one of the metabolites (sulfonamide), Accinelli and Koskinen (2007) studied the environmental fate of sulfonamide antimicrobial agents in soil and reported that concentration of sulfonamide up to 10,000 μg kg^{-1} had no effect on the soil microbial community. Therefore, it is likely that the detected concentrations of

sulfonamide in flucarbazone-treated soil samples of Beaverlodge (Table 5.2; 2.3 and 4.0 μg kg^{-1} of sulfonamide was chemically extracted from 20 and 30 g a.i. ha^{-1} flucarbazone treated soils, respectively) were not high enough to negatively influence the native rhizobial and total microbial populations. The report by Accinelli and Koskinen (2007) was one of the few published reports available which dealt with the influence of AHAS herbicides or their metabolites on soil bacterial inhabitants, and it warrants further field studies into the potential influence of AHAS inhibitors on soil microbial populations.

5.5 Conclusions

A mustard bioassay and chemical extraction were conducted on the field soil samples to test for the presence of residual flucarbazone one year after its application in the field. Even though the chemical extraction detected the presence of residual flucarbazone and its metabolite, the concentrations were not high enough to cause root growth inhibition in mustard. As field pea was found to be more tolerant to a given concentration of residual flucarbazone than mustard, it is not surprising that no effects of residual flucarbazone on the plant development were observed in our field experiments (Chapter 4).

Despite the presence of various concentrations of chemically extractable flucarbazone and its metabolite, no statistically significant differences in the native rhizobial and total soil microbial population were detected among the flucarbazone-treated field soil samples. Based on the results from the field experiment, bioassay, and chemical extraction, it can be concluded that the soil residual flucarbazone and its metabolite were not present at high enough concentrations in Goodale and Beaverlodge soils to negatively influence the growth of host plants or the nodulation success of inoculum rhizobia in year 2.

When standard curves were constructed for the bioassay using the two different soils, it was noted that the phytotoxicity of flucarbazone of a given concentration was much greater in soil with less SOM and clay contents. Such findings emphasize the influence of soil characteristics on carryover injury caused by herbicides with residual properties. It can be speculated that if the soil with low SOM and clay content is combined with environmental conditions that favour herbicide persistence in soil, the producers may observe carryover injury from flucarbazone in their rotational field pea crops. In order to minimize such risk, producers are advised to adhere to the herbicide application instructions provided by the manufacturer, and be mindful of the environmental and soil conditions that may reduce the rate of herbicide degradation.

6 SUMMARY AND CONCLUSION

This study was conducted to elucidate the effect of residual AHAS-inhibiting herbicide, flucarbazone, on the success of inoculation in field pea production in western Canada. Specifically, this study tested the following null hypothesis: *The presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia*.

Firstly, a growth chamber experiment (Chapter 3) was conducted to reveal the negative influence of flucarbazone on plant growth and nodulation. Field pea was grown in soils spiked with a wide range of flucarbazone concentrations (0 to 40 μg kg^{-1} range in 5 μg kg^{-1} increments). The concentration range of flucarbazone application was chosen to ensure that sufficient herbicide would be present in at least some of the treatments to cause a negative impact, which would allow us to establish the approximate critical level of soil flucarbazone concentration at which negative effects on field pea growth and nodulation would be evident.

The findings from the growth chamber study clearly demonstrated the susceptibility of field pea to the presence of flucarbazone in soil where the lowest concentration of flucarbazone amendment (5 μg kg^{-1}) significantly reduced the plant growth (i.e., above-ground and root biomass). Further addition of flucarbazone (10 to 40 μg kg^{-1}) did not result in incremental changes in plant growth parameters. Similar to the plant growth parameters, a considerable reduction in nodule formation (i.e., total number and weight) was observed in the flucarbazone-treated plants (5 and 10 μg kg^{-1} spiked plants). When the soil flucarbazone concentration was increased to 15 μg kg^{-1}, the plant was still alive, yet nodulation was eliminated. Such devastating influence of

flucarbazone on nodulation was manifested in the source of N incorporated into plant tissue where the analysis of natural abundances (δ) of the stable isotope pair $^{15}N/^{14}N$ in the above-ground biomass indicated that the control plants with abundant nodules met their N demand via symbiotic N_2-fixation. The flucarbazone-treated plants with minimal or no nodules satisfied N requirements by taking up N from the soil. Therefore, it was speculated that if the environmental conditions are such that the concentration of residual flucarbazone in soil approaches 5 μg kg^{-1} (approximately 25% of recommended field application rates), a producer would likely notice a significant reduction in plant growth, nodulation, and N benefits from field pea production.

The findings from the growth chamber study led to the field experiment (Chapter 4). The focus of the field experiment was to examine whether field-aged, one-year-old residual flucarbazone negatively influence field pea production and nodulation as observed in the growth chamber study. Specifically, an attempt was made to elucidate whether flucarbazone applied at the field recommended rate during wheat production in a given year would persist into the following season at high enough concentrations to negatively influence both the growth of subsequently cropped field pea and the inoculation success. In order to simulate flucarbazone carryover during crop rotation, wheat was grown in 2005 (year 1) at field sites in Saskatchewan and Alberta, and it was treated with three rates of flucarbazone-sodium (0, 20, 30 g a.i. ha^{-1}) to control weeds, where the recommended field rate is 30 g a.i. ha^{-1} (≈ 20 μg kg^{-1}). The control plots that received no flucarbazone were treated with non-residual herbicides containing no AHAS inhibitor at the field recommended application rates. At each site, all treatments were replicated four times, and arranged in a randomized complete block design.

When various parameters of field pea production and nodulation were observed at two field locations, no statistically detectable differences originating from the use of flucarbazone were identified. Specifically, statistically detectable differences were not

found in: shoot and root weight, seed yields, nitrogenase activity (ARA), total nodule number and weight, and N concentration and $\delta^{15}N$ of above-ground plant material. Such results implied the absence of soil residual flucarbazone one year after application, or the amount of flucarbazone carried over into the following season was too low to influence plant and nodule development. Regardless of the actual concentrations of the residual flucarbazone in the test plots, these findings from the field experiment led to the conclusion that flucarbazone applied at the field recommended rate during wheat production in a given year would not persist into the following season to negatively influence field pea production and inoculation success, if soil and environmental conditions promote flucarbazone degradation.

To confirm the findings of the field experiment, the soil samples collected from the field sites were analyzed for flucarbazone (Chapter 5). Firstly, the concentration of soil residual flucarbazone and its metabolites in the soil samples was determined using a mustard bioassay and chemical extraction. The chemical extraction conducted on the flucarbazone-treated field soil samples detected various concentrations of residual flucarbazone and its metabolite; however, the concentrations were not high enough to inhibit root growth of mustard grown in these soils (i.e., mustard bioassay; Chapter 5). As field pea was found to be more tolerant to a given concentration of residual flucarbazone than mustard, the results from bioassay and chemical extraction supported the results of the field experiment where field pea grown in the flucarbazone-treated field plots showed no signs of herbicidal influence in any of the parameters observed (Chapter 4).

Secondly, despite the lack of detectable herbicidal influence on plant growth and nodulation in the field experiment (Chapter 4), it was still possible that the native rhizobial population was influenced by the use of flucarbazone either through direct contact of herbicide with native rhizobia at the time of herbicide application and/or by

the potential presence of trace amount of residual flucarbazone which may be enough to negatively influence the native rhizobial population. Since the population of native rhizobia can compete against inoculum rhizobial strains for infection sites and influence inoculation success, the size of native rhizobial population also was determined in the soil samples collected from all three treatments (0, 20, and 30 g a.i. ha^{-1} flucarbazone-treated plots) prior to seeding pea in year 2. The results from the plant infection MPN (for native rhizobial population) and CFU plate count (for total viable soil microbial population) confirmed that the use of flucarbazone in year 1 up to the recommended field rate and the resultant presence of residual flucarbazone and its metabolites in year 2 (as detected by chemical extraction) had no detectable influence on the native rhizobial and total soil microbial population size at the time of field pea production in year 2 (Chapter 4).

The interpretation of the findings from the present study with regards to the hypothesis statement (*The presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia*) depends on the concentration at which residual flucarbazone is present in soil. As previously mentioned, if the soil residual flucarbazone concentrations approach 5 μg kg^{-1} (approximately 25% of recommended field application rates), plant growth and nodulation would be reduced significantly and overall nodulation of field pea by inoculum rhizobia would be affected. On the other hand, at low enough concentrations as in the present field study (Chapter 4), it is safe to assume that the presence of residual flucarbazone in soil does not affect nodulation of field pea by inoculum rhizobia. This speculation is based on the observably comparable inoculation success of field pea among the three treatments (0, 20, and 30 g a.i. ha^{-1} flucarbazone-treated plots) as indicated by the lack of statistically detectable differences in plant growth, grain yields, nodulation, nitrogenase activities, plant tissue N content, and the size of native rhizobial population among the three treatments.

Having speculated the absence of residual herbicidal impact on nodulation success by inoculum rhizobia, a possibility was recognized that the proportion of nodules occupied by the inoculum rhizobial strains may differ among the three treatments without affecting any of the parameters measured for the present study. To address such uncertainties, an attempt was made to genetically identify the origin of nodule occupants using a molecular microbiological method (Chapter 3 and 4). Although the sequencing results revealed the potential overestimation on the nodulation success of strain R_2 (Section 4.4.3) the visual classification of strain R_1 appears accurate, and it was consistently found by the growth chamber study (Chapter 3) and the field experiment (Chapter 4) that the nodulation success of strain R_1 is unaffected by the use of flucarbazone. Therefore it appears safe to conclude that the use of flucarbazone up to the field recommended application rate did not influence the proportion of nodules occupied by the inoculum rhizobial strains.

Overall, the findings from the growth chamber study (Chapter 3), field experiment (Chapter 4), and field soil analyses (Chapter 5) confirmed that flucarbazone applied at the field recommended rate during wheat production would not persist into the following season at high enough concentrations to negatively influence field pea growth, grain yields, and the measured parameters of successful nodulation, if soil and environmental conditions promote flucarbazone degradation, as in the present field study. These findings, together with the genetic analysis of nodule occupants, point to the conclusion that the amount of soil residual flucarbazone found in this study did not interfere with the inoculum rhizobia from fulfilling their tasks of ensuring nodulation and N_2 fixation for the host plant, field pea. From the view point of the producer, the findings from the present study also signify that in order to minimize the risk of carryover injury from flucarbazone while maximizing yields and the N benefit of inoculation, it is advisable to adhere to the herbicide application instructions provided by

the manufacturer, and be particularly mindful of the environmental and soil conditions that may reduce the rate of herbicide degradation.

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8 APPENDICES

APPENDIX A: Recipes for the micro- and macro-nutrient solutions, and flucarbazone spiking solutions

Micro-nutrient stock solution:

Molybdenum (Mo)	with $NaMoO_4 \cdot 2H_2O$	0.363 g	
Boron (B)	with H_3BO_3	2.059 g	
Manganese (Mn)	with $MnSO_4 \cdot H_2O$	3.692 g	Dissolve in 4 L
Zinc (Zn)	with $ZnSO_4 \cdot 7H_2O$	4.222 g	distilled H_2O
Copper (Cu)	with $CuSO_4 \cdot 5H_2O$	0.566 g	

Macro-nutrient stock solution:

Phosphorus (P)	with $Ca(H_2PO_4)_2 \cdot H_2O$	29.300 g	
Sulfur (S)	with K_2SO_4	58.480 g	Dissolve in 4 L
Potassium (K)	with KCl	41.495 g	distilled H_2O

Flucarbazone spiking solutions:

Prepare a stock solution by dissolving 0.01 g of flucarbazone (99.1% pure) in 50 mL of methanol, and by adding distilled H_2O to attain the final volume of 1 L. By using the stock solution, prepare a series of spiking solutions containing flucarbazone at concentrations of 0, 0.5, 1, 2, 3, and 4 mg L^{-1}.

For example, in order to prepare a pot containing 1.2 kg of 75 % FC soil with nutrients and herbicide amendments at soil flucarbazone concentration of 40 µg kg^{-1}, above mentioned micronutrients stock solution (20 mL), macronutrients stock solution (20 mL), and flucarbazone stock solution (4 mg L^{-1} concentration solution at 12 mL) were mixed in a beaker, distilled H_2O was added to attain the appropriate final solution volume necessary to reach 75% FC moisture in 1.2 kg of a given soil. The solution mix was then applied to the soil in a pot, and the soil was thoroughly mixed in the plastic bag used to line the pot to ensure even distribution of the nutrients and herbicide throughout the pot.

APPENDIX B: An example contingency table for the statistical analysis of successful nodule colonization by the inoculum rhizobia

For each treatment, four replicated pots were present with each pot containing two plants. From each replicated pot, six nodules were analyzed for each region (crown or distal), and the presence of the inoculum strain was recorded in terms of a fraction as in "X/6" where X = number of nodules containing an inoculum strain. For example, the results from the genetic analysis of the distal nodule samples collected from 5 and 10 μg kg^{-1} flucarbazone-treated plants were recorded as below:

Treatment (μg kg^{-1})	# of nodules colonized by inoculum rhizobia			
	Rep.1	Rep.2	Rep.3	Rep.4
5	2/6	4/6	1/6	4/6
10	3/6	2/6	3/6	3/6

To conduct Pearson's chi-square (χ^2) test, the above table was expressed in the form of a contingency table as below:

Treatment (μg kg^{-1})	Replication	Colonization by inoculum rhizobia:		Total
		Yes	No	
5	1	2	4	6
5	2	4	2	6
5	3	1	5	6
5	4	4	2	6
10	1	3	3	6
10	2	2	4	6
10	3	3	3	6
10	4	3	3	6
Total	—	22	26	48

APPENDIX C: FAME profiling experiment

Summary of the preliminary FAME experiment and its outcome

To complete a part of Chapter 3 and 4, a simple and rapid method was required to identify the origin of rhizobial nodule occupants as either inoculum or non-inoculum rhizobial strains. Prior to the adaptation of the molecular microbiological method as described in Chapter 3 and 4, the use of FAME (fatty acid methyl ester) profiling was considered. As described in Section 2.5.1, fatty acid profiling utilises the variability in the composition and proportion of bacterial membrane lipids. Since such lipid biomarkers differ among microbial genera and species (Spiegelman et al., 2005), a plan was conceived to construct reference database entries of fatty acid profiles on inoculum rhizobial strains to which fatty acid profiles of unknown nodule occupants would be compared to identify nodule occupants as either inoculum or non-inoculum rhizobial strains. However, it has been reported that the overlap of fatty acid characteristics can occur at subspecies level among microorganisms of genetic variant or subtype (i.e., strains), in which case accuracy of identification may decrease (Stead et al., 2000).

Thus, a preliminary experiment was conducted with various known strains of rhizobial pure cultures to determine if FAME analysis would provide sufficient resolution to distinguish inoculum rhizobia from non-inoculum native strains. More specifically, well-characterized rhizobial pure cultures were used to create reference database entries of fatty acid profiles. These profiles were then compared against each other to examine if samples of different rhizobial strains would exhibit sufficient difference in Euclidian Distance (ED) when plotted on a dendrogram. Simply stated, a dendrogram is the visual representation of the relatedness among the tested rhizobial strains based on their fatty acid composition, and ED refers to the distance in two dimensional space between any two strains on a dendrogram (Flury and Riedwyl, 1988). It has been reported that strains linking at ≤ 25 ED units are likely to belong to the same genus and those linking at ED < 10 or < 6 are likely to be the same species, or strains,

respectively (Sasser, 2001). Based on these guidelines proposed by Sasser (2001), the preliminary experiment was conducted as described in the following sections to examine if FAME analysis of different rhizobial strains would result in a dendrogram where they are linked at ED > 6.

Examination of the dendrogram (figure not shown) revealed that except for the chickpea rhizobia (*Rhizobium* sp. *Cicer* strain USDA4794), all four *R. leguminosarum* rhizobia tested (i.e., *R. leguminosarum* bv *viceae* strains R_2 and ATCC10004 and *R. leguminosarum* bv *phaseoli* strains ATCC14482 and USDA2667) were linked at ED < 6, indicating that FAME profiles of these strains were too similar to be identified as distinct strains. Since strain level identification was necessary for the present study, the use of FAME analysis was not explored further, and molecular biological method was adopted instead.

Materials and methods

Bacterial strains

The rhizobia used in this experiment included two named strains of field pea rhizobia (*Rhizobial leguminosarum* bv *viceae* strains R_2, and ATCC10004), two strains of bean rhizobia (*R. leguminosarum* bv *phaseoli* strains ATCC14482 and USDA2667) and one strain of chickpea rhizobia (*Rhizobium* sp. *Cicer* strain USDA4794). *R. leguminosarum* bv *viceae* strain R_2 was one of the two strains present in a commercial peat inoculant (NitraStik-C®; EMD Crop BioScience, WI) which was used in the field experiment (Chapter 4). The pure culture of the inoculum strain was generously provided by EMD Crop BioScience. Rhizobia denoted with ATCC and USDA refer to the well-characterized pure culture collections obtained from American Type Culture Collection

and United States Department of Agriculture, respectively, which are maintained at the University of Saskatchewan soil microbiology laboratory.

Extraction of cellular fatty acids

Each of the aforementioned bacterial pure culture was streaked on modified tryptone yeast extract agar plates (MTY: tryptone , 5.0 g; yeast extract, 3.0 g; $CaCl_2$, 0.87 g; mannitol, 1.0 g; agar, 15.0 g; distilled water, 1.0 L) (Beringer, 1974), and grown at 28 °C for 48 h. A standard procedure established by Sasser (Sasser, 2001) was used in extraction and preparation of bacterial fatty acids. From each MTY plate, 40 mg of bacterial cells (the second and third quadrant streak of growth) were collected by using an inoculation loop, and the cells were placed at the bottom of a screw cap test tube. Enough MTY plates were prepared to have nine replicated tubes per strain so that nine sets of replicated fatty acid profile data would be available to create one profile entry for each strain.

To saponify the bacterial cells, 1 mL of sodium hydroxide solution (sodium hydroxide, 45 g; methanol, 150 mL; distilled water, 150 mL) was dispensed into each test tube, vortexed for 10 s, heated in a boiling water bath for 5 min, vortexed again for 10 s, then incubated in a boiling water bath for 25 min. When the tubes were cooled, methylation was conducted to form fatty acid methyl ester (FAME). Two millilitres of hydrochloric acid solution (6.0 N hydrochloric acid, 325 mL; methanol, 275 mL) was added, vortexed for 10 s, incubated for 10 min at 80 °C, and cooled immediately in a water bath. Following methylation, 1.25 mL of organic solvent (1:1 hexane:methyl tertiary butyl ether) was added to each tube, gently shaken for 10 min, and aqueous phase was pipetted out, leaving FAME in the solvent phase. Finally, the solvent remaining in the tube was washed by gently shaking for 5 min with 3 mL of sodium hydroxide solution (sodium hydroxide, 10.8 g; distilled water 90 mL), and the organic

phase was transferred into a gas chromatography (GC) vial for analysis. FAMEs in the organic phase were analysed with a Hewlett-Packard 5890 Series II GC fitted with 25 m of phenyl methyl silicone capillary column and a flame ionization detector (FID). The extract was split 100:1 prior to passing through the column to the FID. The column was programmed to change from 170 to 270 °C at a rate of 5 °C min^{-1} to allow separation of the fatty acids and comparison to known standards for naming the peaks. The GC was attached to a microprocessor containing Hewlett-Packard Microbial Identification System software (Mendala, 1990).

Construction of a reference FAME database

Based on the GC analysis data revealing the unique combination and proportion of various fatty acids present in each FAME sample, a reference FAME profile entry was created for each rhizobial strain using MIDI Library Generation Software developed by MIDI/Hewlett-Packard Microbial Identification System (Mendala, 1990). A reference FAME profile entry for each strain consisted of nine replicated FAME profiles.

Data analysis

Once the reference FAME profile entries were created for each strain, a dendrogram was constructed to examine if rhizobia of different strains would be linked at ED > 6. A dendrogram was constructed by using a built-in function of the aforementioned MIDI Library Generation Software.