UREIDE ACCUMULATION IN FABA BEAN (*Vicia faba* L.)

A Thesis Submitted to the College of Graduate Studies and Research

In Partial Fulfillment of the Requirements

For the Degree of Master of Science

In the Department of Plant Sciences

University of Saskatchewan

Saskatoon

By

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ABSTRACT

Faba bean (*Vicia faba* L.) is a cool season crop that uses symbiotic biological nitrogen fixation to obtain atmospheric nitrogen (N), a limiting macronutrient, for growth and maintenance of the plant. Most cool season legumes like faba bean transport N from the nodules as amides, which are metabolized in destination tissues. Ureide metabolism is a catabolic process that produces N rich compounds from purine rings. Many warm season legumes such as soybean and common bean produce ureides (allantoin and allantoate) in their root nodules and then use these molecules to transport fixed nitrogen from root to shoot. Non-ureide exporting plants such as faba bean also produce ureides in normal purine recycling whereby these compounds may play a role in response to abiotic stress. This research aims to examine possible differences in ureide metabolism across genotypes and to assess the role of ureides in response to water limitation. In field grown faba bean, total ureides were found in highest concentrations in leaf tissue, followed by reproductive parts, stems, and nodules, but were not found to differ significantly among genotypes. Ureide concentrations varied throughout the growing season, decreasing over time as the plants reached physiological maturity. A water limitation experiment of faba bean grown in a controlled environment showed that faba bean accumulated the ureides allantoin and allantoate after six to eight days of water limitation when all data were pooled. However, no consistent trend was observed comparing results by genotype, and inoculated versus non-inoculated plants. Overall, results indicate that faba bean likely does not use ureides to transport symbiotically fixed N and that ureide accumulation in field grown plants is most likely in response to abiotic stress or remobilization of purine N from senescing tissues.

ACKNOWLEDGEMENTS

I would like to thank my supervisor's Dr. Christopher Todd and Dr. Rosalind Bueckert for their guidance and support throughout my research and graduate studies. I particularly would like to thank Dr. Todd for his encouragement, advice, assistance, and persistence in pushing me to keep going until the very end. I would also like to thank Dr. Bueckert for giving me my start in graduate studies as a Master's student and providing me with the plant material and data to perform various aspects of my research project. Thank you both for your financial contribution to my studies. I would also like to acknowledge the financial support I received from the Robert P. Knowles Scholarship.

I would also like to thank my committee members, Drs. Gordon Gray, and Kirsten Bett for their input in my research and thesis. A big thanks to Dr. Eric Lamb who provided guidance and direction in the statistical analysis of my data. I would also like to thank all my labmates Matthew Munson, Jodi Souter, Solmaz Irani, Veronica Duran, and Maryam Nourimand for their help and encouragement throughout my graduate studies. Thank you to Matt and Jodi for your training and guidance in the lab, especially with the HPLC. Thank you to Solmaz for your companionship and help in my lab work. Thank you to Veronica for training me in the molecular techniques of the lab. Thank you as well to all other students who helped me with my research project, James Dawson, Jenalee Michkolz, Adrian Reetz, and Melanie Jean.

Thanks as well to my family members; Mom, Dad, Grandma, brother, and especially my sister for your encouragement in finishing my degree. For my son Wyatt, I hope having your mom go school for many years of your life will inspire you to one day study anything and everything that interests you because learning new things is always good. And lastly, but definitely not in the least, I would like to thank my husband Amr El Zawily for pushing me and helping me every step of the way throughout my graduate studies. Thanks for believing in me and giving me the support and encouragement I needed to finish this degree. Thank you for all your help in bettering my understanding of science and molecular techniques as well as pushing me to try my hand at teaching. You not only helped me with the scientific aspects of my research but you helped me on an emotional and supportive level. And I'm so grateful that graduate studies led me to meet you.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ACT	Actin
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AIC	Akaike's Information Criterion
AAH	Allantoate amidohydrolase
ALN	Allantoinase
NH_3	Ammonia
NH_4^+	Ammonium
NH_4NO_3	Ammonium nitrate
ANOVA	Analysis of variance
Asn	Asparagine
BLASTN	Basic Local Alignment Search Tool for nucleotides
BLASTX	Basic Local Alignment Search Tool for proteins
BNF	Biological nitrogen fixation
H_3BO_3	Boric Acid
$CaCl_2{\cdot}2H_2O$	Calcium chloride dihydrate
$CoCl_2{\cdot}6H_2O$	Cobalt chloride hexahydrate
cDNA	Complementary deoxyribonucleic acid
$CuSO_4{\cdot}5H_2O$	Copper sulfate pentahydrate
CDC	Crop Development Center
cv.	cultivar
df	Degrees of freedom
denDF	Denominator degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
N_2	Dinitrogen
K_2HPO_4	Dipotassium phosphate
K_2SO_4	Dipotassium sulfate

DM	Dry matter
DWt	Dry weight
EST	Expressed sequence tags
EV	Evapotranspiration
FeNaEDTA	Ferric sodium ethylenediaminetetraacetic acid
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate
glm	General linear model
GS/GOGAT	Glutamine synthetase/glutamate-oxoglutarate aminotransferase
GLN	Glutamine
GLU	Glutamate
GLY	Glycine
GA	Glyoxylate
GMP	Guanosine 5'-phosphate
ha	Hectare
HPLC	High Performance Liquid Chromatography
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
HIU	5-hydroxyisourate
HIUHase	Hydroxyisourate hydrolase
IMP	Inosine monophosphate
kg	Kilogram
kV	Kilovolts
KH_2PO_4	Monopotassium phosphate
lme	Linear mixed model
LB	Lysogeny broth
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
Mn	Manganese
$MnSO_4 \cdot H_2O$	Manganese sulfate monohydrate
µF	Microfarad
M	Molar/Molarity
nm	Nanometer

nmol	Nanomole
$NiCl_2 \cdot 6H_2O$	Nickel chloride hexahydrate
NiR	Nitrite reductase
NR	Nitrate reductase
NF	Nitrogen fixation
OHCU	2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoleine
PPFD	Photosynthetic photon flux density
KFeCN	Potassium ferricyanide
PCR	Polymerase chain reaction
PRPP	5-phosphoribosyl-1-pyrophosphate
ROS	Reactive oxygen species
g	Relative centrifugal force
RT-PCR	Reverse transcriptase-polymerase chain reaction
rpm	Revolutions per minute
Ser	Serine
NaOH	Sodium hydroxide
$NaMoO_4 \cdot 2H_2O$	Sodium molybdate dehydrate
$NaHPO_4 \cdot 7H_2O$	Sodium monophosphate heptahydrate
SWC	Soil water capacity
H_2SO_4	Sulfuric acid
Tg	Teragram
TAE	Tris-acetate EDTA
UGlyAH	Ureidoglycine aminohydrolase
UAH	Ureidoglycolate amidohydrolase
UO	Uricase oxidase
XDH	Xanthine dehydrogenase
XMP	Xanthine monphosphate
$ZnSO_4 \cdot 7H_2O$	Zinc sulfate heptahydrate

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Faba bean (*Vicia faba* L.) of the Family Fabaceae, commonly called broad bean or horse bean, is an ancient pulse crop originating in the Mediterranean, Central Asian, and Middle Eastern regions where it has been cultivated for approximately 8,000 to 10,000 years (Zohary and Hopf, 1973). Faba bean is of two types, the smaller seeded faba bean (*Vicia faba* var. *minor*) and the larger seeded Chinese variety (*Vicia faba* var. *major*) (Jensen et al., 2010). Faba bean is grown for human consumption as a vegetable, dried or green, fresh or canned, and even roasted, and is used as an animal feed or green manure crop (Bond et al., 1985). World production during 2007-2011 was 4.1 to 4.8 million tonnes per year, with China the largest producer, accounting for 40 to 45% of the world faba bean production, followed by Ethiopia, Australia, France, and Egypt (FAOSTAT, 2013). World area of faba bean sown each year has considerably declined, about 56% since 1962; however, since the average yield has nearly doubled in that time from 1 to 1.8 tonnes per hectare, the decrease in production has only been 20% (FAOSTAT, 2008). The global average yield of faba bean is quite variable by country and from year to year, with the average being 1.8 tonnes per hectare in 2006 (FAOSTAT, 2008). Yields in Canada have been more stable than other countries from the years 1982 to 2005, with grain yields hovering around 2100 kg dry matter (DM) per hectare (FAOSTAT, 2008).

Faba bean is a long day, indeterminate, cool season legume with a high N-fixing capacity compared to other cool season pulse crops such as chickpea, lentil, and pea (Herridge et al., 1994). The amount of dinitrogen (N_2) fixed by faba bean ranges from 78 to 330 kg N ha^{-1}, or an average of 200 kg N ha^{-1}, where 59 to 92% of N present in the crop is derived from biological nitrogen fixation (BNF) (Herridge et al., 1994). This is higher than the amount of N_2 fixed in chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), and field pea (*Pisum sativum*), which range from 3 to 141 kg N ha^{-1}, 1 to 192 kg N ha^{-1}, and 17 to 244 kg N ha^{-1}, respectively (Herridge et al., 1994). In order to produce the world's grain and oilseed

crops, about 100 teragrams (Tg) of N are needed every year (Herridge and Rose, 2000). Of that, 17 Tg of N is supplied through BNF by leguminous crops. Since the world's population is constantly increasing, and agriculture must supply the world with food on an ever decreasing land base, it is favorable to include leguminous crops into a crop rotation, in that the N residue left in the soil can be used by the subsequent crop, helping to alleviate fertilizer use (Beck et al., 1991). Faba bean is an economically important pulse crop that has the ability to fix N, where the greater its ability to fix N, the greater its ability to increase yields and reduce farmers' reliance on N fertilizer, dependence on fossil fuels, as well as reduce greenhouse gas emissions (Duc et al., 1988; Nemecek et al., 2008; Peoples et al., 2009).

Nitrogen is a limiting factor in plant growth and development and can be difficult for plants to obtain from the environment because it is only found in limited amounts in forms available to plants. Therefore, some plants have developed mechanisms to obtain unavailable N from the atmosphere by a biological symbioses with N-fixing free-living soil bacteria in order to fix dinitrogen (N_2). In legumes, the nitrogen-fixing bacteria infect the plant roots and the plant forms root nodules around the bacteria, providing an environment with low oxygen required in order to reduce N_2 by the enzyme nitrogenase (Mylona et al., 1995). During this process ammonium is produced by the reduction of N_2 by the bacteria. Ammonium is then incorporated into organic compounds by the plant and is exported from the root nodules to the rest of the plant for further metabolism (Mylona et al., 1995). In turn, plants provide photosynthate carbon (C) to the bacteria, which serves as their energy source. This exchange of C for N is critically important for maintaining this symbiotic relationship between plant and bacteria.

Generally, once ammonium is assimilated in the root nodules of plants, it is converted first into the amide or cyclic amino acids asparagine (Asn) and glutamine (Gln). Certain species designated as warm-season or tropical legumes, such as soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), mung bean (*Vigna radiata*), and pigeon pea (*Cajanus cajan*) export N as ureides, or cyclic amino acids with functional urea groups, primarily allantoin and allantoate (Schubert, 1986). The other legume known as; cool-season or temperate legumes, include lupin (*Lupinus* spp.), field pea, alfalfa (*Medicago sativa*), white clover (*Trifolium* spp.), and faba bean are called amide-

exporters, exporting N in amide form (Schubert, 1986). Both amides and ureides are exported to the various sink tissues within the plant and are degraded or metabolized through a series of enzymatic reactions. In plant leaves, allantoin is metabolized by the enzyme allantoinase into allantoate. Allantoate is further metabolized by enzyme-catalyzed reactions to ultimately release ammonia into sink tissues to be re-assimilated by plant cells for use in protein and nucleic acid synthesis and metabolic processes.

Studies have shown that the activity of N fixation in some legumes becomes sensitive to drought or water-limiting conditions (Sinclair and Serraj, 1995). Cowpea and soybean, both tropical legumes, were found to be drought sensitive, showing a higher concentration of ureides in the xylem sap following water limitation. This accumulation of ureides was thought to act as a feedback inhibition to nitrogen fixation causing nitrogen fixation to slow. However, recent research in common bean showed that ureide accumulation was present in both N-fixing and non-fixing plants (Diaz-Leal et al., 2012). Therefore, N fixation is not affected by ureide accumulation and is uncoupled from synthesis of ureides in the root nodules (Alamillo et al., 2010). This ureide accumulation has been shown to be under genetic control under water limiting conditions, with increased transcript levels of allantoinase (Alamillo et al., 2010). Ureides have also been shown to be involved in a stress response whereby high levels of ureides were found in stressed plant tissues (Brychkova et al., 2008). In more recent research, genes linked to abiotic stress resistance and abscisic acid (ABA) production were linked to ureide metabolism, where increased ureide accumulation activates ABA production, which is responsible for mediating a stress response in plants (Watanabe et al., 2013).

1.2 The current study

Faba bean has been demonstrated to be an indeterminate growing cool season legume that utilizes amide-export of N from root to shoot (Beck et al., 1991; Herridge et al., 1994). However, when testing faba bean samples for total glyoxylate derivatives or total ureides in 2010, ureides were found in all sampled plant partitions; leaf, stem, reproductive, and nodule (Rosalind Bueckert, unpublished). This could suggest that faba bean may be a ureide-exporter of N and not an amide-exporter as previously stated, or it could utilize both forms of N transport as in chickpea (Thavarajah et al., 2005). The

purpose of this study was to assess whether ureide metabolism plays an important role in transporting fixed N in faba bean, using total ureide analysis through spectrophotometric determination of total ureides, HPLC analysis of the individual ureide breakdown products (allantoin, allantoate, and uric acid), and a gene expression study of genes involved in the ureide catabolism.

1.2.1 Objectives

This research investigated the role of ureide accumulation in faba bean. At the onset of my research, my primary hypothesis was that faba bean utilizes ureide transport as well as amide transport and that under water limiting conditions, ureides will accumulate in plant tissues. A second hypothesis I addressed was ureide accumulation in response to abiotic stress independent of N fixation. The specific objectives were:

1. To quantify the amount of total ureides in field grown genotypes to detect any significant differences in ureide concentrations among genotypes.
2. To analyze and quantify the amount of individual ureide breakdown products (allantoin, allantoate, and uric acid) from faba bean plants subjected to a controlled water limitation experiment during the vegetative growth stage.
3. To identify allantoinase and allantoate amidohydrolase gene sequences in faba bean to facilitate future gene expression studies.

1.3 Literature review

1.3.1 Biological nitrogen fixation

In economically important leguminous plants (Subfamily Faboideae), BNF is a key mechanism by which N is obtained from the abiotic environment (Amarante et al., 2005). Biological nitrogen fixation begins with signaling in the rhizosphere between free-living soil bacteria, mostly *Rhizobium* spp., and plant roots by Nod factors (Lindström et al., 2002). Plant roots release compounds of Nod gene expression, secretions of phenolic compounds (flavonoid and/or isoflavonoid), and the bacteria respond by sending out Nod factors (oligiosaccharides) that alter the plants growth pattern (Peters and Verma, 1990; Sanjuan et al., 1992). The altered plant growth traps the endosymbiont, usually by an infection thread, which curls around the bacteria (Pueppke, 1986). The bacteria multiply

and divide in the infection thread and then penetrate the root cell layers where the bacteria are released in membrane-bound vacuoles called symbiosomes (Day et al., 2001). The bacteria then differentiate in the symbiosomes to form bacteriods. Within infected root cells, the bacteria enclosed in the bacteroids fix gaseous N with the help of the enzyme nitrogenase and ATP, produced by bacterial respiration, to reduce N_2 to ammonia (NH_3) (Brown and Dilworth, 1975). This is a symbiotic relationship whereby the plant supplies photosynthate in the form of carbohydrates or sugars to the bacteria to be used as C skeletons for molecules that incorporate N, as well as for bacterial respiration that produces ATP, while the bacteria fix atmospheric nitrogen into ammonia, providing the plant with N required for growth and maintenance (Long, 1989).

Measurements of BNF have been used as an indicator of sustainable agriculture by comparing the amount of N fixed by the crop, relative to the amount of N already in the soil, to the amount left over in the soil after the crop has been harvested. Faba bean and other cool season pulse crops such as chickpea, lentil, and field pea, are N-fixing crops and can be used in a crop rotation to help maintain the soil N balance. Studies have shown that when only the crop grain is harvested and the straw or stubble is left on the field, the amount of soil N increases. In one study by Beck et al. (1991), cool season legumes were grown to assess their impact on soil N balance. Overall results showed that faba bean fixed 144 kg N ha^{-1} more than it exported in seed production leading to the highest net soil gain in N of all the crops grown. Given the results of this study, faba bean can potentially supply N to the subsequent crop and contribute to a positive soil N balance leading to more sustainable farming practices.

1.3.2. Nitrogen metabolism

1.3.2.1 Introduction to nitrogen fixation in legumes

Once N is fixed in the root nodules of legumes by symbiotic bacteria, the plant must assimilate the fixed N in order to be utilized for maintenance and growth. The exported products of N assimilation differ among legumes, where certain cool season legumes such as field pea, faba bean, chickpea, lentil, lupin alfalfa, and clover transport N from root to shoot primarily as amides, namely Asn and Gln (McClure and Israel, 1979; Streeter, 1979; Schubert, 1986). The second type of N transported from root to shoot occurs in warm

season legumes such as soybean, cowpea, common bean, and mung bean. These species transport N primarily as ureides, namely allantoin and allantoate or cyclic amino acids with bound urea groups, from root to shoot (McClure and Israel, 1979; Streeter, 1979; Schubert, 1986; Sprent et al., 1987). These legumes with differing types of N export have been termed either 'amide-exporters' or 'ureide-exporters.'

Nodule growth in legumes can also classified according to the type of N export, where amide-exporting legumes have an indeterminate type of nodule growth with an open branched vascular system that restricts water flow (Schubert, 1986). Because of this restricted water flow, amides are found in higher molar concentrations in the xylem sap than are ureides (Schubert, 1986). Ureide-exporting legumes have a determinate type of nodule growth where a series of closed loops form from vascular strands fused apically that connect with the main vascular system at the nodule base. This forms a closed loop system that provides reduced water flow resistance, allowing for more water to flush the products of N fixation from root to shoot, which helps transport the less soluble ureides in ureide-exporting legumes (Schubert, 1986).

Plant roots also assimilate N by absorbing nitrate from the soil solution by different membrane transporters (Crawford and Forde, 2002). Once nitrate is assimilated into the plant it gets converted into nitrite with the help of the enzyme nitrate reductase (NR). Following this, nitrite is reduced into ammonium by nitrite reductase (NiR) (Crawford, 1995). Ammonium can also be absorbed by plant roots through ammonium transporters. Once ammonium is present in the plant roots, it is incorporated into the glutamine synthetase/glutamate-oxoglutarate aminotransferase (GS/GOGAT) cycle since high levels of ammonium are toxic to plant tissues (Miflin and Lea, 1976). Application of mineral N actually inhibits N fixation in legumes compared to plants that are not treated with mineral N (Svenning et al., 1996), causing the plant to utilize the applied N rather than products of N-fixation.

1.3.2.2 Amide export

Amide-export is the most common type of N export, which is typically found in cool season legumes such as alfalfa, clover, chickpea, lentil, white lupin, field pea, and faba bean (Schubert, 1986). Cool season legumes are those legumes that prefer cooler growing

temperatures and can even be grown over the cooler winter months in warmer climates. Faba bean is considered a cool season legume in that it has indeterminate growth and utilizes the amide export of N, with over 75% of the N in the xylem sap comprised of the amides Asn and Gln (Beck et al. 1991; Herridge et al. 1994; Pate et al., 1979; Peoples et al., 1987).

In amide-exporting legumes, ammonium (NH_4^+) is released into the cytosol and plastids of the infected root cells where glutamine synthetase and NADH-dependent glutamine 2-oxoglutarate amidotransferase (NADH-GOGAT), also named glutamate synthase, are present (Miflin and Lea, 1976; Miflin and Cullimore, 1984). Glutamine synthetase catalyzes the addition of ammonium to glutamate (Glu), an ATP-dependent reaction, where N from the ammonium is incorporated into the amide position of glutamate (Miflin and Cullimore, 1984). The hydroxyl group on the amino acid residue glutamate has an NH_2 added to it to produce glutamine, which is then exported out of the nodule. Glutamine produced by this amidation reaction enters the plastid, where it can be acted on by GOGAT, using the reducing power of NADPH or reduced ferrodoxin, to produce two glutamate molecules by the transfer of the secondary amino group from glutamine to alpha-ketoglutarate (Coruzzi, 2003). One glutamate molecule is recycled back into the cytosol, which can be combined with ammonia in the glutamine synthetase reaction to produce glutamine again. One glutamate molecule regenerates the glutamine/glutamate cycle, and one is incorporated with oxaloacetate (OAA) to regenerate alpha-ketoglutarate and produce aspartate (Asp) by aspartate aminotransferase (Reynolds et al., 1981; Coruzzi, 2003). Aspartate can then be catalyzed by asparagine synthetase to add an amide group to the carboxyl of the amino acid glutamine, producing asparagine, which can then be exported out of the nodule (Lea and Fowden, 1975; Forde and Lea, 2007). These amides are the main form of N transported to the aerial plant parts through the xylem sap where they are transaminated, transamidated, and metabolized (Pate et al., 1979; Muñoz et al., 2011).

1.3.2.3 Ureide export

Ureide-exporters are typically warm season, tropical legumes including soybean, common bean, cowpea, pigeon pea, and mung bean (Pate et al., 1980). In ureide-export, the

majority of the N transported out of the nodule is comprised of the ureides allantoin and allantoate, and a small portion as the amides, asparagine and glutamine, as well as nitrate from non-fixing nitrate uptake by plant roots (Streeter, 1979; Peoples et al., 2002). Ureide production begins in the infected cells of root nodules, where N_2 is fixed in the bacteroid by bacteria into ammonia (Streeter, 1979). Ammonia is transported out of the bacteroid into the cytosol where it is incorporated into glutamate to form glutamine by the enzyme glutamine synthetase (Herridge et al., 1978). Glutamine can then be immediately transported out of the nodule as glutamine, or it can be incorporated with alpha-ketoglutarate, in the plastid, to produce two glutamate molecules. Glutamate and aspartate are then used in the production of purine biosynthesis.

A number of enzymes catalyze the reaction of purine nucleotide synthesis using NADPH as reductant and ultimately generating xanthine. Xanthine is shuttled out of the plastid to the infected root cell cytosol. Here xanthine is broken down by xanthine dehydrogenase to produce uric acid, or urate, a purine (Triplett et al., 1980). Uric acid is transported to the peroxisomes of uninfected cells and is further broken down by urate oxidase with the addition of O_2 to produce allantoin (Tajima and Yamamoto, 1975). Allantoin can then be transported out of the uninfected cells into the xylem sap or can be further broken down by allantoinase into allantoate and then be transported out of the uninfected cells into the xylem stream (Hanks et al., 1981). Nitrate is taken up by plants roots and is transported through the xylem stream; however, this comprises a minimal amount of the xylem sap. These ureides then travel up the xylem sap to the aerial plant parts to be used for growth and development in biomass and seed production (Matsumoto et al., 1976; Alamillo et al., 2010). In plant leaves, ureides are broken down into ammonia, CO_2, and glyoxylate, where NH_3 is again used in the GS/GOGAT cycle (Todd and Polacco, 2004). When ureide-exporting plants are actively fixing N, the xylem sap is dominated by ureides; however, when the plant is supplied with N in the form of soluble nitrate or ammonia fertilizer, the transport form of N switches from ureide form to amide form (Matsumoto et al., 1977; Herridge, 1982).

1.3.3 Costs of nitrogen fixation

The overall nitrogen fixation equation is (Dixon and Kahn, 2004; Seefeldt et al., 2009):

$N_2 + 8\ H^+ + 16\ ATP + 8e^-$ ---> $2\ NH_3 + H_2 + 16\ ADP + P\acute{\imath}$ [Eq. 1.1]

Fixed N requires C skeletons and reducing power in order to be assimilated by plants, therefore, there is a cost associated with N assimilation in addition to direct ATP consumption (Todd et al., 2006). Ureides are the products of purine degradation or oxidative catabolism, where purine rings are either synthesized *de novo* or nucleic acids are turned over and recycled into ureides (Todd et al., 2006). Urate, derived from xanthine dehydrogenase activity, or purine degradation, in the bacteria infected cells of the nodule, is transported to uninfected cells and is oxidized by microbodies to form allantoin. Ureides require a C skeleton in which to attach N groups onto, in a ratio of one C molecule required for every one N molecule present (C:N=1:1) (Layzell et al., 1979). The cost of producing a C skeleton requires the energy source of ATP. It has been estimated that five ATP are required to produce one ureide molecule of allantoin or allantoate (Todd et al., 2006). For amide-exporting legumes, the cost to produce amides is higher, with 12 ATP required to produce one Asn molecule. The C:N ratio is also less efficient for amide production, with 2:1 C:N per Asn, and 2.5:1 per Gln. Therefore, the cost of ATP energy needed for N assimilation in ureide-exporting legumes is half the cost of amide exporting legumes. Ureide exporters are more conserving of photosynthate than amides since their C:N ratio is 1:1.

Even though ureide production has been calculated according to ATP requirements, where ureides are less expensive to produce compared to amides, five ATP per allantoin or allantoate versus 12 ATP per Asn, ureide production does have a cost associated with it. This cost is associated with the purine biosynthesis taking place in the nodule, as well as in the release and re-assimilation of ammonia in the leaf (Todd et al., 2006). The four C ureido-derived NH_4^+ molecules that are released in the leaf require four C skeletons, alpha-ketoglutarate molecules incorporated via the GS/GOGAT cycle, for every ureide molecule transported. The C component of the ureides, the 2-ureido groups, is converted into CO_2 and glyoxylate. Glyoxylate likely enters the photorespiratory cycle and CO_2 is re-fixed to generate ammonia and CO_2 and aids in the formation of phosphoglycerate. In the amide-transporting pathway, the amide N of Gln or Asn is mobilized by transamidation reactions.

Alpha-N, in glutamate or aspartate, is already on a C skeleton, therefore, only one C skeleton is required for each amide molecule to be metabolized. Consequently, the C and energy requirement needed to assimilate the products of N_2 fixation in the destination tissue are greater in ureide-export than in amide-export.

1.3.4 Measurements of bacterial nitrogen fixation

Various methods have been developed and used to measure BNF. These techniques include: the dry matter yield method (DM); total N difference method; nodule observation; acetylene reduction assay (ARA); xylem-solute technique; ureide xylem sap method; and ^{15}N methodologies. The ^{15}N methodologies include: $^{15}N_2$ gas; the use of ^{15}N enriched fertilizers or substrates-Isotope dilution method (ID); A-value method (AV); and the natural abundance method (NA) (Hardarson and Danso, 1993). Over the years, methods of measuring BNF have improved to become more accurate and sensitive. The ^{15}N natural abundance and ureide techniques are the most commonly used methods (Peoples et al., 2002).

The level of ureides relative to the amount N in the xylem sap ([ureide-N/ureide-N + nitrate-N] x 100) has been used as an indicator of the level of symbiotic N fixation taking place between plants and bacteria in warm season or tropical legumes. In a study by Herridge (1982), on the tropical legumes soybean and cowpea, up to 80% of the N transported within the plants was in the ureides; allantoin, allantoate, in fully symbiotically N-fixing plants. Herridge (1982) found that when these legumes were fertilized with N in solution, BNF did not occur and minimal ureide levels, less than 15% of xylem solutes were present in the xylem sap. He found a linear relationship between the relative abundance of ureides and symbiotic dependence of the plants on N fixation when examining the N solutes of the shoot axis and root nodules. Soybean and cowpea plants that were fed nitrate solution showed higher levels of mostly Asn and some Gln and minimal levels of ureides, therefore, the more N fixation occurring, the higher the level of ureides found within the plant. This was calculated using the total N uptake/plant compared to the N_2 fixed/plant or N_2 fixed per plant as a percentage of total plant N. Herridge (1982) also found that completely symbiotic soybean plants produced the most nodular tissue and as plants were exposed to increasing levels of nitrate fertilizer, the nodular tissue decreased significantly

in size. Ureide levels were measured using a spectrophotometric assay that quantifies ureides as the phenylhydrazine derivative of glyoxylate (Vogels and Van Der Drift, 1970). Herridge surmised that since ureide concentration changes due to the level of BNF occurring between the plant and bacteria, the ureide concentration relative to N concentration within the plant could be used as a screening tool to select for high N fixing crops or cultivars (1982). This screening could be used in breeding programs to improve crop yields.

1.3.5 Ureide metabolism

1.3.5.1 Ureide synthesis

Ureides, or cyclic amino acids with nitrogen or urea groups attached, are produced from Gln after the incorporation of ammonium into the GS/GOGAT cycle, where Gln is used to synthesize ureides in warm season legumes as a means to transport the N fixed in the root nodules to the shoot of the plant to be released as ammonia and used for the plant's growth and development. The production of ureides originates from purine turnover from RNA and DNA known as the salvage pathway or from *de novo* synthesis in the root nodules (Schubert, 1986). Plants are also able to carry out this important process of converting purines into inorganic N as nitrate and NH_4^+, the all-important macronutrient required for proper growth and development in plants. Therefore, the main purpose of purine catabolism in all plants (which has not been demonstrated experimentally) is to provide N for the growth of new plants parts such as leaves, flowers, and seeds by remobilizing N from older plant tissues (source) to younger developing tissues (sink) through purine recycling (Zrenner et al., 2006).

In the infected cells of the root nodules, plastids and mitochondria synthesize purines using the amino acid Gln (Smith and Atkins, 2002). Phosphoribosylamine (PRA) is formed from glutamine and 5-phophoribosyl-1- pyrophosphate (PRPP) (Zrenner et al., 2006). Following this is a ten-step process, involving different enzymes amino acids, and CO_2 to produce *de novo* synthesis of inosine monophosphate (IMP) from the previously mentioned PRPP. Inosine monophosphate is then transformed into xanthosine monophosphate (XMP) by the IMP dehydrogenase enzyme. Xanthosine monophosphate is

then converted into xanthosine by 5' nucleotidases and xanthosine is transformed by inosine/guanine nucleosidase into xanthine (Zrenner et al., 2006).

The degradation pathway that all purine bases undergo begins with the common intermediate xanthine. Xanthine is oxidized by xanthine dehydrogenase (XDH) into urate or uric acid in the plastids of infected root cells. Following this, urate is converted into 5-hydroxyisourate (HIU) by an oxidation reaction catalyzed by uricase, reducing oxygen to H_2O_2 (Modrić et al., 1992; Kahn et al., 1997). It was found that HIU is unstable in aqueous solution and that at room temperature and at neutral pH, it degrades non-enzymatically to yield allantoin forming a racemic mixture of allantoin and HIU. A novel enzyme purified from soybean root nodules, called hydroxyisourate hydrolase (HIUHase) was isolated by Sarma et al. (1999), which catalyzed the hydrolysis of HIU (Sarma et al., 1999). This unstable product yielded 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoleine (OHCU) by C-NMR analysis. Spontaneous decarboxylation of OHCU produces a racemic mixture of allantoin, but *in vivo*, *S*-allantoin is normally the product of urate breakdown, which indicates that an enzyme catalyzes the decarboxylation of OHCU. The dual enzyme, allantoin synthase, has been recently demonstrated to hydrolyze HIU to OHCU and OHCU to allantoin in Arabidopsis (Lamberto et al., 2010; Pessoa et al., 2010). This enzyme has two domains, one with an HIU hydrolyzing domain and another with an OHCU decarboxylzing ability. In summary, xanthine undergoes an oxidation reaction catalyzed by XDH to form urate in the cytosol, moves to the peroxisome where it is oxidized to HIU by uricase, and HIU is converted to *S*-allantoin through the intermediate OHCU by the dual-functional enzyme allantoin synthase. From this stage allantoin is transported to the endoplasmic reticulum for further degradation (Werner and Witte, 2011). The following figure shows the main steps of ureide synthesis and catabolism that occurs in plants (Fig. 1.1).

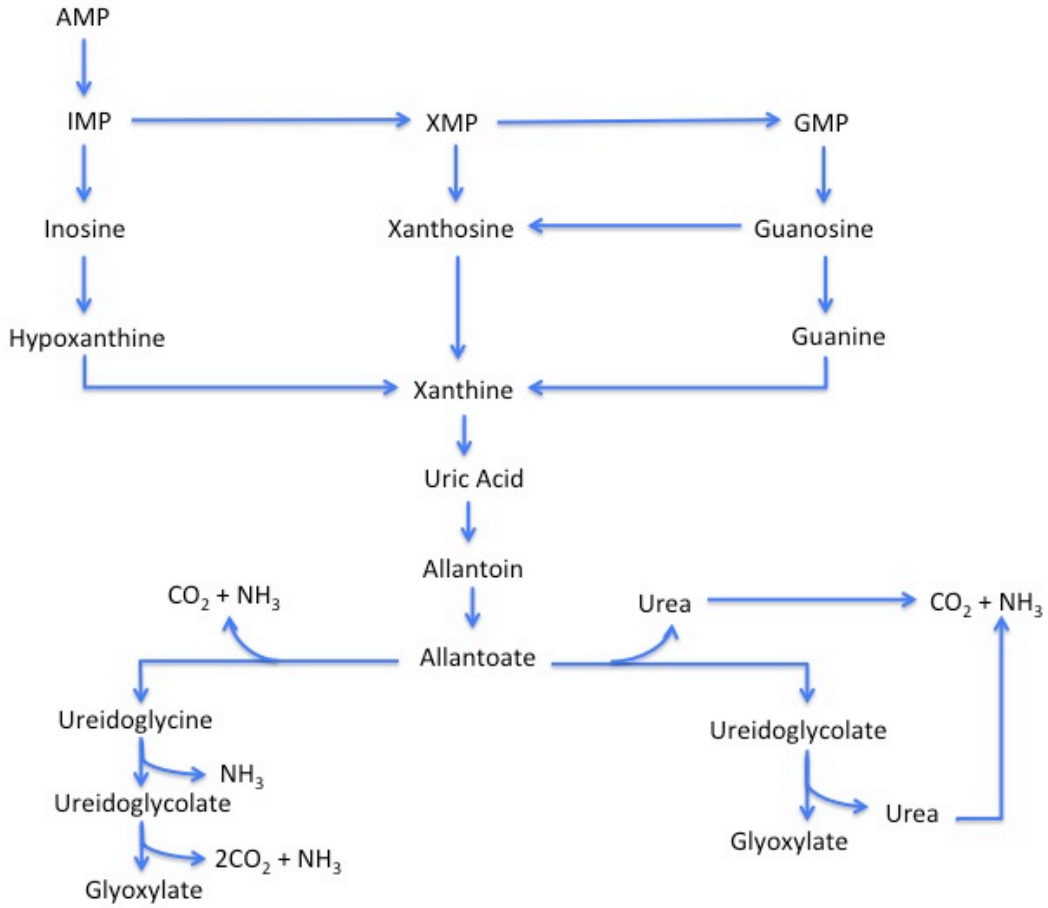

Figure 1.1. The main steps of ureide synthesis and catabolism in plants. Metabolic components shown are: 1. adenosine monophosphate (AMP), 2. inosine monophosphate (IMP), 3. xanthosine monophosphate (XMP), 4. guanosine monophosphate (GMP). Adapted from Zrenner et al., 2006.

1.3.5.2 Ureide catabolism

The next step in ureide catabolism involves the breakdown of allantoin to allantoate by the enzyme allantoinase in the endoplasmic reticulum (Werner et al., 2008) (Fig. 1.2). For the complete utilization and assimilation of N to occur, the two-ureido (N-containing) groups of allantoate must be converted to ammonia. In plants, the enzyme that hydrolyzes allantoate is allantoate amidohydrolase (AAH) to ureidoglycine, NH_3, and CO_2 (Winkler et al., 1985; Todd et al., 2006; Werner et al., 2008).

In French bean and chickpea, an alternative enzyme has been purified and characterized named ureidoglycolate urea-lyase, which also has ureidoglycolate degrading ability, converting ureidoglycolate into glyoxylate and urea (Muñoz et al., 2001; Muñoz et al., 2006). Ureidoglycine, the intermediate in the former reaction, is quickly broken down enzymatically by ureidoglycine aminohydrolase (UGlyAH) into ureidoglycolate and ammonia, a product that is always present in the breakdown of allantoate (Werner et al., 2010; Serventi et al., 2009). This product ureidoglycolate, is then degraded through the intermediate, hydroxyglycine, to produce CO_2, ammonia, and glyoxylate by the enzyme ureidoglycolate amidohydrolase (UAH) (Werner et al., 2010). Glyoxylate is then metabolized most likely by an enzyme of the photorespiratory pathway to form glycine and serine (Winkler et al., 1987), while ammonium is most likely recaptured by glutamine synthetase in the cytosol. The overall ureide catabolic equation is as follows:

Uric acid (5C, 4N) ---> $3CO_2$ + $4NH_3$ + glyoxylate (2C) [Eq. 1.2]

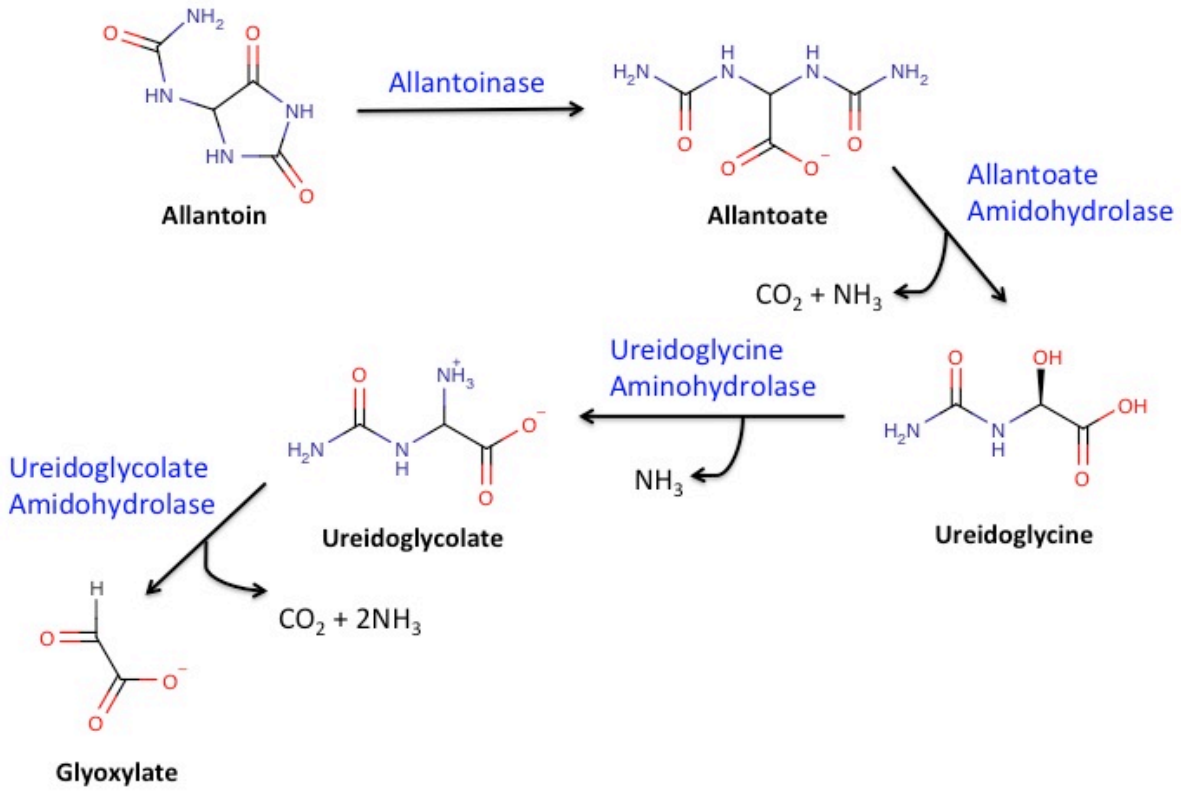

Figure 1.2. The main steps of ureide catabolism showing ureide breakdown by the respective enzymes into the end product glyoxylate, CO_2, and ammonia in plant leaves. Adapted from Werner and Witte, 2011.

1.3.6 Ureides and stress conditions

1.3.6.1 Ureides under water limiting conditions

The role ureides play in response to stress has been a long debated point in the understanding of ureide catabolism. Ureide catabolism during stress conditions has been thought to impact the rate-limiting step in N fixation. Under water deficit in soybean, ureides accumulate mainly in the leaves of the plant while N fixation rapidly declines (Sinclair and Serraj, 1995). Nodules also show increased levels of reduced C and N. This led to the conclusion that N fixation down-regulation due to drought stress may be a result of a decreased shoot requirement for fixed N. As N fixation is down regulated, transpiration slows as stomata close.

Sinclair and Serraj (1995) have shown that amide-exporting legumes do not show as much sensitivity to drought and that their transpiration rate and overall level of N fixation does not slow as significantly. Some believe that the accumulation of ureides in plant leaves acts as a negative feedback mechanism that inhibits N fixation in the root nodules, and that cultivars that do not accumulate ureides in leaf tissue are those that appear to have greater drought tolerance by prolonging N fixation (Charlson et al., 2009). The hypothesis is that a lack of aerial C skeletons leads to an inability to accept N in ureide form. This lack of C skeletons can be rectified with an input of CO_2. The xylem sap has been shown to change composition as well. King and Purcell (2005) have shown that under water-limiting conditions, the amount of Asn increases significantly, of all the xylem sap amino acids (King and Purcell, 2005). Bacanamwo and Harper (1997) have postulated that an increase in Asn acts as a negative feedback mechanism for N-fixation and that as its concentration increases ureide catabolism decreases (Bacanamwo and Harper, 1997). They also speculate that the C: N balance as well as a plant's ability to assimilate the products of ureide breakdown may be the cause of nitrogenase activity being down regulated.

In a study by Amarante et al. (2005) on amide- and ureide-exporting legumes, as well as non-legumes, amino acid concentrations changed in all plants when subjected to stress conditions. Flooding of N fixing plants resulted in impaired N fixation where the level of Gln in the xylem sap fell from 20% to 0.7 to 2.7%. Along with this, the Asn in the xylem sap showed a marked decrease while aspartate (Asp) showed a large increase and ureide levels, the direct products of N fixation, decreased as well. The increased Asp to Asn ratio is likely attributable to altered Asn synthetase activity within root nodules. Reduced substrate availability, reduced enzyme levels, or both may affect this enzyme. Asn synthetase catalyzes the conversion of aspartic acid to Asn through an amidation reaction using the N donor Gln. Following N_2 or NO_3 reduction, Glu is considered the immediate ammonia assimilation product, serving as a link between N_2 fixation, NO_3 assimilation, and Asn synthetase. Amarante et al. (2005) found that this change in amino acid composition was present in both symbiotic and non-symbiotically fixing plants and in both amide- and ureide-exporting legumes as well as non-leguminous plants.

More recent research has shown that in soybean, ureide and amino acid accumulation in below ground plant organs occurs with no change in transpiration when plants are

drought stressed and N fixation declines (Gil-Quintana et al., 2013). Gil-Quintana et al. (2013) found that ureide accumulation in soybean was related to a decrease in the ureide degradation rate and not due to their biosynthesis. Using proteomic analysis they found that amino acid metabolism, cell growth, carbon metabolism, as well as protein synthesis were the most altered processes in root nodules. These results suggest a local inhibition and regulation of N fixation and a minor role that ureides play in N fixation inhibition in soybean. Another study using *Medicago truncatula* measured amino acid content and N fixation activity in drought-stressed plants and found that amino acids accumulate in the roots before a drop in evapotranspiration was measured (Gil-Quintana et al., 2012). They concluded that drought results in a local inhibition of N fixation as well, which leads to an overall amino acid accumulation throughout the plant body, and that this increase in amino acids is independent of any decrease in evapotranspiration. Their conclusions suggest that no single amino acid is responsible for N fixation inhibition but an overall amino acid accumulation, resulting in a feedback inhibition of N fixation.

A study by Diaz-Leal et al. (2012) on the crop common bean looked at the developmental effects of ureide levels mediated by regulation of allantoin (Díaz-Leal et al., 2012). They found ureide levels were roughly equal in N fixation bean leaf and shoot tissue to that found in plants provided with an inorganic N source during vegetative growth. However, at the onset of flowering, ureides began to accumulate, which was accompanied by an increase in the gene expression and enzyme activity of allantoinase. This implies that ureide levels are regulated developmentally by tissue-specific activation of allantoate synthesis through catalysis by the enzyme allantoinase. They found high levels of ureides present in non-nodulated plants at early and late developmental growth stages. These results suggest that N is remobilized from older plant leaves to younger leaves given the sharp increase in ureides in the leaves and shoots of the plants during the later developmental growth stage of mid-pod fill in both N-fixing and non-fixing plants.

1.3.6.2 Ureides and oxidative stress

Ureide metabolism is important in nitrogen recycling and mobilization from nucleic acid or protein turnover. Brychkova et al. (2008) have demonstrated that in Arabidopsis, ureides play an important role in cell protection against oxidative stress and could play an

integral role in the alleviation of oxidative stress due to adverse growing conditions (Brychkova et al., 2008). Alamillo et al. (2010), suggest that for ureidic legumes under stress conditions, ureide metabolism is used in nitrogen mobilization when leaf senescence is triggered to remobilize N from the leaves to the rapid generation of seeds, especially during pod fill.

Brychkova et al. (2008) used Arabidopsis mutants lacking either one of two *XDH* genes, termed *Atxdh1* and *Atxdh2*. They subjected wild types (WT) and mutant plants to extended dark treatment (6 days) and found that WT plants had increased transcripts of ureide catabolism, with greatly increased levels of allantoin and allantoate. The mutant *Atxdh1* however, showed an accumulation of xanthine and early symptoms of senescence. The mutant had increased degradation of chlorophyll, widespread cell death, as well as increased transcript levels related to senescence. Upon the recovery period and re-exposure to light, mutant lines compared to WT plants had increased levels of reactive oxygen species (ROS) and a higher rate of mortality. Interestingly, upon the addition of the ureides allantoin and allantoate, the levels of ROS and cell death decreased. This finding suggests that ureides may actually act as ROS scavengers and suggests that AtXDH1 enzyme activity plays a significant role in dark-induced catabolism of purines through the production of ureides. Brychkova et al. (2008) hypothesize that ureides perform two functions; to serve as cellular protectants against ROS and to act as transportable metabolites, used in other biosynthetic pathways. Their results show that aging, extended dark exposure, and purine catabolism all show similarities to senescence, and that in all of these instances there is a transition from assimilation of nutrients to metabolic turnover due to an increase in the purine catabolic recycling activity where the enzyme AtXDH1 plays an important role.

Recent investigations by Watanabe et al. (2013) demonstrated research on ureides and plant stress and their role in abiotic stress tolerance using Arabidopsis plants. They used three knockout mutants, two for ALN, *aln-1* and *aln-2*, one for AAH, *aah,* and lastly one for XDH, *xdh1,* where all gene knockouts encoded for enzymes catalyzing steps in ureide catabolism. Their results showed that Arabidopsis plants exposed to drought and osmotic stress have enhanced tolerance to these stresses in the mutant plants for *aln* and *aah* compared to WT. The mutant plants were able to grow better on plates with high osmotic

stress and had significantly more biomass than the WT plants. The ALN mutation was found to increase ABA levels and change the plant's response to ABA as well, making it more adaptable and viable. The *aln* mutant showed up-regulated gene expression of those genes involved in the stress response, where an accumulation of ALN resulted in increased stress-responsive gene expression of rate-limiting enzymes in ABA synthesis and degradation. Their overall results suggest that under stress conditions in plants, allantoin degradation is a crucial part of purine catabolism (Watanabe et al., 2013). This metabolite acts as a housekeeping intermediate in a response to stress allowing plants to respond swiftly to any environmental changes.

CHAPTER 2. MATERIALS AND METHODS

2.1 Faba bean field experiment

2.1.1 Plant material

Faba bean (*Vicia faba*) genotypes used for this research were of two types, colored (purple) flowers or white flowers, where those that had colored flowers contained condensed tannins while white flowering plants did not contain condensed tannins. The original faba bean genotypes were obtained from various breeders (Table 2.1).

2.1.2 Experimental design

The faba bean field experiment was designed to analyze fifteen different genotypes of faba bean (Table 2.1) to assess whether they differed in terms of yield, total nitrogen content, nitrogen fixation, as well as the amount of ureides or total glyoxylate derivatives by genotype. Plants were grown over two field seasons and two different field locations in Saskatchewan, Canada and sampled at three different times during the growing season; anthesis (flowering), mid-pod fill, and physiological maturity (when the plant leaves begin to yellow and pods are ripening). A completely randomized block design with replications (blocks) was used and the 15 genotypes were grown at both Rosthern and Skarsgard, Saskatchewan during the summer of 2009 and 2010. Plants were sampled from two 25 cm rows at the different growth stages. Plants were partitioned into leaves, stems, reproductive parts (at anthesis: flowers; at mid-pod fill: flowers and/or pods together; and at physiological maturity: reproductive parts were flowers + pods without seeds, and seeds collected alone), and nodules for further analyses. Plant tissues were dried at 60°C for 48 h and ground to pass through a one mm screen. Plant roots were collected from the same plants sampled at each growth stage and were dried as described above and photos were taken of each root sample. Dried nodules were then picked off of each root sample and ground to a fine powder. The amount of total ureides expressed as glyoxylate derivatives of faba bean were analyzed.

Table 2.1 The 15 different genotypes of faba bean used in the field trial based on their tannin content, flower color, and breeding origin.

Genotype	Breeder	Flower Color/Tannins
CDC Fatima	Crop Development Center (CDC), University of Saskatchewan	Purple/present
CDC SSNS_1	Crop Development Center (CDC), University of Saskatchewan	Purple/present
Divine	Agri-Obtentions, France	Purple/present
FB6_83	Crop Development Center (CDC), University of Saskatchewan	White/absent
FB22_10	Crop Development Center (CDC), University of Saskatchewan	White/absent
FB25_56	Crop Development Center (CDC), University of Saskatchewan	White/absent
FB34_2	Crop Development Center (CDC), University of Saskatchewan	White/absent
FB34_7	Crop Development Center (CDC), University of Saskatchewan	White/absent
Florent	NPZ (Norddeutsche Pflanzenzucht) Hans-Georg Lembke KG, Holtsee, Germany	White/absent
Imposa	Limagrain Advanta Nederland BV, Rilland, Netherlands	Purple/present
Melodie	Agri-Obtentions, France	Purple/present
NPZ4_7540	NPZ (Norddeutsche Pflanzenzucht) Hans-Georg Lembke KG, Holtsee, Germany	White/absent
NPZ5_7680	NPZ (Norddeutsche Pflanzenzucht) Hans-Georg Lembke KG, Holtsee, Germany	Purple/present
Snowbird	Innoseeds B.V., Vlijmen, Netherlands	White/absent
Taboar	HA van Mierlo, Globe Seeds B.V., Vlijmen, Netherlands	Purple/present

2.1.3 Quantification of total ureides

Approximately 35 mg subsamples of the dried faba bean tissues were used in a spectrophotometric assay to calculate the amount of total ureides as glyoxylate (GA) derivatives based on the procedure of Vogels and Van der Drift (1970), with reduced volumes. Samples were prepared for the assay by adding 1 mL of 0.2 M NaOH to each sample in a microcentrifuge tube. Tubes were heated in a water bath for 15 min at 100˚C, then cooled to room temperature (rt) and centrifuged at 13,000 *g* for 3 min. Tubes were frozen and stored for later use. For analysis, tubes were thawed and a 200 µL aliquot of

supernatant was pipetted into a 16 x 150 mm glass test tube and 1 mL of distilled H_2O was added. Next, 200 µL of 0.5 M NaOH was added to each sample, followed by 200 µL of 0.65 M HCl. Tubes were heated to 100˚C for 4 min in a boiling water bath and then allowed to cool to rt. Next, 200 µL of 0.4 M phosphate buffer (Na_2HPO_4 – KH_2PO_4, pH 7.0) was added to each tube, followed by 200 µL of 23 mM phenylhydrazine-hydrochloride and tubes were mixed and left at rt for 5 min. Samples were cooled on ice for 5 min and then 1 mL of ice cold concentrated HCl was added. Tubes were removed from the ice bath and 200 µL of 51 mM potassium ferricyanide (KFeCN) was added. Samples were mixed and left at rt for 15 min. A standard curve using GA (5, 10, 50, 100, 200, 300 µM) was generated in parallel using the same procedure described above. After 15 minutes absorbance of both samples and standard curve tubes at 522 nm were measured using a UV-Visible spectrophotometer (Agilent 8453 UV-Vis Diode Array System, Santa Clara, CA, USA). The concentration of each sample was determined based on the standard curve obtained for each set of samples analyzed. Data are expressed as µmol of GA equivalents per gram of dry weight (DWt) of tissue.

2.1.4 Data analysis for field experiment

Statistical analyses of total ureide content as total glyoxylate derivatives were performed using R software, version 2.14.0. Parametric analyses were carried out using the linear mixed model or 'lme' to determine significant differences by genotype and plant partition for ureides with a confidence of 95% or a $p<0.05$. In analyzing the ureide concentration by quantifying the total glyoxylate derivatives in the faba bean samples, a series of statistical analysis were employed to analyze the data.

2.1.4.1 Initial data exploration

During initial data exploration, histogram and quantile-quantile (qqnorm) plots were used on the response variable ureides, for all samples from the three different growth stages; anthesis, mid-pod fill, and physiological maturity, to determine if the data were normally distributed or not. Using a histogram of ureides at growth stage mid-pod, the data showed a histogram skewed to the left, which meant the data did not follow a normal distribution (Appendix A). Since the data were not normally distributed, the data were log

(natural logarithm) transformed and a histogram of the transformed data showed a bell-shaped curve with a normal distribution (Appendix A).

To account for the different factors in this experiment, all parameters considered as random effects were those that influence the variance of the response variable, ureides. The factors that influence the mean ureide concentration are genotype and plant partition and were set as fixed effects in the model. There were several sources of non-independence in this experiment such as location and year, which were combined as one factor in this analysis into location x year (location x year interaction), block, genotype, and plant partition. This experiment had a nested design where samples were taken at different levels within a hierarchy. For example, each plant partition was sampled within the plots according to genotype, while genotype was nested within the blocked design, and each block was nested within location x year interaction. The model was fit with the hierarchy of random effects or factors; genotype, block, and location x year nested within each other. The data contained categorical explanatory variables, which meant a linear model was not recommended to use on such a dataset. Therefore, a linear mixed effects model or 'lme' was chosen to analyze the dataset containing these nested categorical explanatory variables to observe the response variable, ureides, with the factors, genotype and plant partition.

2.1.4.2 Model selection

Initially, a general linear model or 'glm' was run for the growth stage anthesis to observe the Akaike's Information Criterion (AIC) value of the data compared to a simple linear mixed model or 'lme' model's AIC value (Appendix B). The AIC is a calculated value indicating the goodness of fit of a model. The lower the AIC value, the better the model fit. AIC for the 'glm' model was 926.7 while the mixed model or 'lme' AIC was 893.3. This drop in AIC is due to adding Block as a fixed factor into the model, which tells us the mixed model is fitting the data better since it is accounting for more of the variation affecting the response variable ureides. Then, a variance components analysis was done, showing that most of the variation in the data was due to plant partition (91.2%), location x year (8.82%), residuals (0.0024%), block (<1%), and genotype (<1%) (Appendix B).

Next, the factors location x year, block, and genotype were set as random factors and genotype x plant partition as fixed factors. Model 1 showed a $p<0.0001$, which means that

one of the values significantly differs from zero. The ANOVA results showed that the p-value was <0.0001 for plant partition but p=0.4822 for genotype, and p=0.9897 for the genotype x plant partition interaction while the AIC was 215.5 (Appendix B). To simplify the model, Model 1, the genotype x plant partition interaction was removed since no significant p-value was returned, and the model was reanalyzed (Model 2). Then the fixed factor genotype was taken out of the model since it had no significant effect and the model was run again (Model 3). Since AIC was lowest in Model 3, and all the non-significant terms were excluded, Model 3 is the best minimally adequate model used to explain the data (Appendix B).

The same process of data analyses was carried out on the growth stage, mid-pod fill, and the statistical results were as follows. The variance in the data contributed 94.8% by plant partition, 5.3% by location x year, 0.0014% by residuals, <1% by Block and <1% by genotype (Appendix C). AIC values show that again the model with the genotype x plant partition interaction removed (Model 2, AIC= 573.6) is a better model than with this interaction included (Model 1, AIC= 653.7), and finally with genotype removed from the fixed effects in the model (Model 3) we see the lowest AIC value (513.9), therefore, this is the minimally adequate model (Appendix C).

Statistical analysis at physiological maturity showed similar results. The variance in the data occurred mostly from plant partition at 97.3%, 2.4% by location x year, 0.0926% by residuals, <1% by block and <1% by genotype (Appendix D). Again, based on the AIC values for the models run, Model 3 was chosen as the minimally adequate model since all non-significant terms were removed and it had the lowest AIC value (Appendix D). The same parameters for model selection were used as previously described.

Again, the 'lme' model was used for analyzing the data for nodules. A model was designed using all the random factors (location, year, block, genotype, and time) and time was set as the fixed factor. The same process of model selection was employed as described above, taking out random factors from the model with each selection and keeping the fixed factor time only. After comparing the AIC value for each model run, the fourth model was chosen with an AIC of 97.1, which had the random factors, time and genotype removed (Appendix E). This indicated that Model 4 is the best minimally adequate model used to explain the data.

2.2 Identification of ureide metabolic genes in faba bean

2.2.1 Plant material

Plant material was obtained from three different growth conditions, either germinated as seedlings, grown in a greenhouse, or grown in a controlled environment in a growth chamber. Faba bean genotypes Fatima and NPZ4-7540 were used for all RNA extraction and cDNA synthesis. Growth conditions varied as described in the following three sections.

2.2.1.1 Seedling tissue

Seeds were germinated using 10 x 15 Anchor brand germination paper (St. Paul, MN, USA) moistened with distilled water, then rolled up and covered with Saran™ Wrap at one end. The opposite end was placed in a beaker of distilled water. Seeds were allowed to germinate for 8 days at 22˚C in the light before the seedlings were collected and separated into hypocotyl tissue and cotyledons and then frozen in liquid nitrogen and stored at -80˚C until required.

2.2.1.2 Greenhouse conditions

Seedlings germinated as described above were transferred to 15 cm pots with ½ Sunshine Grow Mix #1 Professional Growing Mix (Sun Gro Horticulture, Seba Beach, AB, Canada) and ½ perlite (Natural Volcanic Perlite, Supreme Perlite Co., Portland, OR, USA) with 3 to 4 seedlings per pot and placed in the Biology Dept. greenhouses over the summer and fall months (July to November) until reaching physiological maturity. Plants were watered to saturation every few days as required and fertilized with regular 20-20-20 Plant Prod™– Water Soluble Fertilizer© (Plant Products Co. Ltd., Brampton, ON, CA) once every two weeks. Plant samples were taken from leaves, flowers, pods, and seeds at 42 d and from leaves, stems, and roots at 106 d and frozen in liquid nitrogen and stored at -80˚C for future use.

2.2.1.3 Growth chamber conditions

Faba bean plants were grown in a controlled environment, growth chamber, according to the following conditions. The growth chamber was kept at a 25˚C daytime

temperature and a 23˚C nighttime temperature with a photoperiod of 16 hours. The humidity was maintained between 50 to 60% with an average photosynthetic photon flux density (PPFD) of 350 µmol m^{-2} s^{-1} at plant height. Seeds from the cultivars NPZ4-7640, Snowbird (both white-colored flowers or zero tannin lines), and CDC-Fatima (colored flowers or tannin containing lines) were germinated for three to four days, as previously described (section 2.2.1.1). Germinated seedlings were transplanted into 15 cm pots and 2 to 3 seedlings were planted per pot in Sunshine Grow mix #1 and were thinned to one plant per pot once seedlings emerged. Plants were fertilized once a week starting at one week after transplanting with a nutrient solution containing 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.31 mM K_2SO_4, 0.5 mM K_2HPO_4, 10 mM FeNaEDTA, 2.5 µM H_3BO_3, 1 µM $ZnSO_4$., 0.1 µM $NaMoO_4.2H_2O$, 0.11 µM $NiCl_2$, 0.15 µM $CuSO_4$, 0.01 µM $CoCl_2$ and 2 µM $MnSO_4$ and 5 mM NH_4NO_3 as a N source to suppress nodule formation.

2.2.2 Identification of *Vicia faba* allantoinase (*VfALN*) and allantoate amidohydrolase (*VfAAH*) gene products

2.2.2.1 Primer design

The known sequence for allantoinase (*AtALN*) in Arabidopsis (gene accession #3706208) was obtained from the The Arabidopsis Information Resource (TAIR) website (www.arabidopsis.org) and was used in a nucleotide BLASTN search at www.phytozome.com, restricted to the subfamily Papilionoideae, to search for similar coding sequences among N-fixing plants. Coding sequences from *Medicago truncatula* (barrel medic or barrel clover) accession #XM_003593471.1, common bean #JG277455.1, and soybean #XM_006597361.1 were aligned using the AlignX feature of Vector NTI® Advance 11 (Invitrogen, Carlsbad, CA, USA) to produce a consensus sequence. The alignment and consensus sequence was used to design primers to amplify a 234 bp fragment of allantoinase in faba bean (*VfALN)* (Table 2.2).

Table 2.2 A list of designed primers used to amplify 234 bp *VfALN,* 529 bp *VfALL* and 474 bp *VfACT* sequences.

Primer Name	Primer Pair Sequence
Vicia faba Allantoinase (*VfALN*)	5'-GTCTGATTCAAGTGCTTCCT-3' 5'-GACTAGTAAGTGGTTGACA-3'
Vicia faba Allantoate Amidohydrolase (*VfAAH*)	5'-TTGAGGTGATTGCATTTAGTG-3' 5'-TAGTTGTACCGGTTCACG-3'
Vicia faba Actin (*VfACT*)	5'-GTTGGATTCTGGTGATGGTGTCAGC-3' 5'-GTTGGTACAAGGGTCCTTAACGAC-5'

A keyword search for allantoate amidohydrolase (*AAH)* from the Phytozome website (www.phytozome.com) was carried out among the nitrogen-fixing plants in the database, and a sequence for *AAH* in soybean was returned (*GmAAH*). The sequence was confirmed to be the correct sequence for *GmAAH* by doing a nucleotide BLAST search on the website, www.**ncbi**.nlm.nih.gov identifying accession #AM773229.1 (Werner et al., 2008). Top hits for similar sequences among other legumes species were returned for *Medicago truncatula, Phaseolus vulgaris*, *Vigna unguiculata*, as well as Arabidopsis. No similar sequences were found for faba bean. Accession #AM773229.1 for *Glycine max*, *Phaseolus vulgaris* #EF650088, and *Medicago truncatula* #XM_003594803 sequences were aligned and a consensus sequence was produced as described above. Primers were designed to amplify a 529 bp fragment of *AAH* in faba bean (*VfAAH*) based on conserved regions (Table 2.2).

2.2.2.2 RNA isolation and quantification and cDNA synthesis

RNA was isolated, according to the protocol in the E.N.Z.A.® Plant RNA Kit (Omega Bio-Tek Inc., VWR International, Mississauga, ON, Canada), from two cultivars of faba bean. Fatima and NPZ4-7540 were selected as representative cultivars either containing or lacking tannins, respectively. RNA was extracted from the hypocotyl tissue of germinated seedlings at 6 d old and from leaves at 48 d. Quantification of RNA was carried out using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and the absorbance ratio at 260/280 nm was measured. cDNA was synthesized using Quanta Biosciences qScript™ cDNA Supermix according to the manufacturer's instructions (VWR International, Mississauga, ON, Canada) and used for polymerase chain reaction (PCR).

2.2.2.3 PCR amplification of *VfALN* and *VfAAH*

To amplify *VfALN* (234 bp), PCR was carried out in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Canada, Mississauga, ON, Canada). The PCR reaction contained: 1) 2 μL 10X PCR Buffer, 2) 2 μL 2 μM dNTPs, 3) 0.5 μL 20 μM forward and reverse primers each, 4) 0.2 μL Taq polymerase, 5) 1 μL hypocotyl cDNA, and 6) 13.8 μL dH_2O, for a total reaction of 20 μL. PCR settings were as follows: 94°C for 2 min; 30 cycles consisting of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s; followed by a final extension step for 10 min at 72°C.

To amplify *VfAAH* (529 bp) the following PCR reaction contained: 1) 2 μL 10X PCR Buffer, 2) 2 μL 2 μM dNTPs, 3) 0.5 μL 20 μM forward and reverse primers, 4) 0.2 μL Taq polymerase, 5) 2 μL leaf cDNA, 6) 12.8 μL dH_2O. A touchdown PCR protocol was used which consisted of the following protocol: 94°C for 2 min, 20 cycles consisting of 94°C for 30 s, anneal for 30 s at an initial temperature of 57°C, decreasing 0.5°C per cycle; 72°C for 90 s; followed by 20 cycles consisting of 94°C for 20 s, 51°C for 30 s, 72°C for 90 s; and a final extension for 10 min at 72°C.

2.2.2.4 Agarose gel electrophoresis and gel extraction

All PCR products were separated on a 1% [weight by volume (w/v)] agarose (BioShop, BioShop® Canada Inc., Burlington, ON, Canada) gel using electrophoresis at 70 volts (V) for approximately 45 min in 1X TAE (Tris, glacial acetic acid, 0.5M EDTA, and water) buffer and visualized using ethidium bromide. Images were captured using a UVP BioDoc-It™ UV gel documentation system (UVP LLC, Upland, CA, USA). PCR products used for gel extraction were run on a 1.5% or 2% (w/v) agarose gel and electrophoresed for approximately 1 h at 45 V. PCR products were gel extracted using the QIAquick® Gel Extraction Kit (QIAGEN Inc. Canada, Toronto, ON, Canada) according to the manufacturer's directions and the purified DNA was visualized on a 2% (w/v) agarose gel to ensure only the band of interest was extracted prior to sequencing.

2.2.2.5 Cloning, isolation of recombinant plasmid DNA and restriction fragment analysis

The PCR fragment for *VfALN* was cloned using the InsTAclone PCR Cloning Kit (Fisher Scientific Canada, Ottawa, ON, Canada), ligating the fragment of interest into the vector pTZ57R/T, with a 3:1 ratio of insert:vector for 1 h at 22°C according to the manufacturer's directions. *Escherichia coli* (strain DH5α) electrocompetent bacteria were transformed by electroporation, by briefly combining 45 µL of bacterial cells plus ~10 µL of ligation mixture in a 1 mm gap electroporation cuvette. Electroporation was carried out at 2.5 kV and a capacitance of 25 µF using the Gene Pulser II electroporation device (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Next, 950 µL of pre-warmed lysogeny broth (LB) media was added to the sample and it was recovered from the cuvette to a clean 1.5 mL microcentrifuge tube and incubated for approximately 60 min at 37°C and 250 revolutions per minute (rpm). After incubation, 10 µL, 100 µL, and 890 µL aliquots of the transformation mixture was plated on the selective LB + ampicillin (AMP, 100 µg mL^{-1}) solid media and spread evenly with a sterile glass rod. The plates were incubated at 37°C overnight. Single colonies were isolated and grown in 5 mL of selective LB liquid medium (AMP= 50 µg mL^{-1}) in tubes at 37°C and 250 rpm overnight.

The E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek Inc., VWR International, Mississauga, ON, Canada) was used to isolate the plasmid DNA from culture according to the manufacturer's recommended protocol. Restriction digest using 1 µL each of the restriction enzymes *XbaI* and *BamHI* and 0.2 µL 10X Tango buffer and 2 µL of plasmid DNA, was carried out to cut the insert out of the plasmid by incubating for 4.5 h at 37°C. The restriction digest analysis was then visualized on a 1% (w/v) agarose gel to see if the correct fragments were present. Two bands were visualized, one at 3000 bp which corresponds to the 2886 bp pTZ57R/T vector and a second band ~230 bp which corresponds to the correct size for the *VfALN* amplified product, data not shown.

The 529 bp DNA fragment for *VfAAH* was purified from an agarose gel and ligated into pJET1 cloning vector provided in the GeneJET PCR cloning kit (Fermentas Canada Inc., Burlington, ON, Canada) according to the manufacturer's recommended protocol. Samples were transformed with *E. coli* (DH5α) and grown on selective media as described previously. Several individual transformants were used to inoculate 5 mL liquid cultures

consisting of LB broth containing 50 µg mL^{-1} ampicillin and grown overnight at 37°C. Plasmid DNA was isolated using the E.Z.N.A® Plasmid Mini Kit I (Omega Bio-Tek Inc., VWR International, Mississauga, ON, Canada) according to the manufacturer's instructions. Purified plasmids were subjected to restriction analysis using *XhoI* and *XbaI* to cut the insert from the vector. Restriction digest products were electrophoresed on a 1% (w/v) agarose gel in TAE buffer. Bands of approximately 3000 bp (the size of the pGeneJet vector) as well as an insert of the approximate size as the putative *VfAAH* PCR fragment ~529 bp were observed by gel electrophoresis, data not shown. Routine molecular biology protocols were carried out according to Sambrook et al. Molecular Cloning: A Laboratory Manual (3rd Ed. 2001, Cold Spring Harbor Laboratory Press, Spring Harbor, New York, USA)

2.2.2.6 Sequencing of *VfALN* and *VfAAH* in faba bean

The plasmid DNA samples were sent to the National Research Council (NRC)-Saskatoon (SK, Canada) for verification of the sequences for both *VfALN* and *VfAAH*. Sequence results were assembled using Contig Express Assembly function in Vector NTI® Advance 11 software to produce a consensus sequence among the results, for each gene. A consensus sequence was generated for both *VfALN* and *VfAAH* and used to search the non-redundant nucleotide database with a BLASTN search (www.ncbi.nlm.gov) to confirm the correct sequence. Bacterial colonies with plasmids carrying the correct sequences for *VfALN* and *VfAAH* were preserved in a 10% glycerol stock and frozen at -80˚C freezer for future use.

2.2.2.7 Amplification of reference housekeeping gene actin

In order to assess changes in gene expression with the genes of interest in this research project, a housekeeping reference gene was chosen to display a gene with constant expression, actin. A search was conducted on www.**ncbi**.nlm.nih.gov website for an actin sequence in faba bean and an expressed sequence tag (EST) sequence, #GQ339767.1 was identified. Primers were designed to amplify a fragment 474 bp in length using the primer design feature of Vector NTI® Advance 11 (Table 2.2). The PCR reaction consisted of: 1) 2 µL 10X PCR buffer, 2) 2 µL 2 µM dNTPs, 3) 0.5 µL 20 µM forward

and reverse primers, 4) 0.2 μL Taq polymerase, 5) 1 μL leaf cDNA, 6) 13.8 μL dH_2O. A fragment was amplified using a PCR protocol of 35 cycles with the following steps; 1) initial heating step for 2 min at 94°C, 2) denaturation for 30 s at 94°C, 3) annealing temperature of 58°C for 30 s, 4) extension step at 72°C for 1 min, 5) steps 2-4 repeated 34X, 6) final extension step for 10 min at 72°C. Faba bean leaf cDNA was used as a template and the sequence of the resulting PCR product was confirmed by sequencing at NRC- Saskatoon.

2.3 Water limitation experiment

2.3.1 Growth conditions and experimental design

Seeds from the genotypes NPZ4-7640 and Snowbird (both white-colored flowers and zero tannin lines) were germinated on germination paper for three to four days as described in section 2.2.1.1. Germinated seedlings of the inoculated treatment were inoculated with *Rhizobium leguminosarium* (TagTeam Granular-Phosphate-solubilizing and nitrogen-fixing inoculant for Field Pea and Lentil, Novozymes BioAg Ltd., Saskatoon, SK, Canada) then transplanted into 15 cm pots and planted two to three seedlings per pot in a Sunshine Grow mix #1 and were thinned to one plant per pot once seedlings emerged. Inoculated plants were fertilized with nitrogen-free nutrient solution once a week starting at one week until the time of water limitation (5 weeks) by watering to saturation with a nutrient solution as mentioned in section 2.2.1.3, but lacking nitrogen. The control or non-N-fixation plants were not inoculated but received the same nutrient solution with the inclusion of 5 mM NH_4NO_3 as a nitrogen source.

Before planting, all pots plus soil were initially weighed (dry weight), then watered thoroughly and allowed to drain overnight. The saturated soil plus pot was weighed again the following day and the difference between the dry and wet soil was calculated as 100% soil water capacity (SWC) for each pot. Then 80% SWC was calculated from the 100% SWC value and each pot was watered every one to two days up to 80% SWC, a calculated weight, until the beginning of the water limitation experiment or until plants were 35 days old. Plants were sampled at the V4 to V5 growth stage where plants were in an established vegetative growth stage, but before the reproductive phase of growth when the plant switches to flowering and producing fruits (Knott, 1990).

Treatments for each genotype were: 1) well-watered (80% SWC) and inoculated, 2) low-watered (20% SWC) and inoculated, 3) well-watered and non-inoculated and 4) low-watered and non-inoculated. Three biological replicates were grown. Pots were arranged in a completely randomized order to minimize the effects of position in relation to light and temperature differences, whereby pots were rearranged once a week to minimize any differences. The growth chamber conditions were the same as mentioned in section 2.2.1.3.

Thirty-five days after planting the faba bean seedlings, the water limitation treatment was started. Low-watered inoculated and non-inoculated plants were allowed to dry down to 20% SWC and then maintained at that level throughout the sampling period. The remaining plants were maintained at 80% SWC. Young leaf tissue was sampled using a #11 cork borer from multi-foliate leaves, that is, those with four leaflets, every two days for 10 days, starting at day 0 of the water limitation period, for a total of six sampling times (Days 0, 2, 4, 6, 8, and 10 of water limitation). Leaf tissue discs weighing roughly 100-300 mg each were weighed and put in 1.5 mL microcentrifuge tubes and frozen in liquid nitrogen immediately and were stored at -80°C for further analysis.

2.3.2 Plant sample preparation for analysis of ureides by HPLC

Ureides were extracted and quantified using high performance liquid chromatography (HPLC) based on a modified protocol developed in the Dr. Chris Todd laboratory [Department of Biology, University of Saskatchewan (Munson, Todd, unpublished)]. Plant samples were preserved at -80˚C until needed for HPLC analysis. Tubes were put into liquid nitrogen directly from the -80˚C freezer, then taken out of liquid nitrogen whereby the leaf samples were ground with a mini mortar and pestle until all the leaf material was finely ground. Ten volumes (greater than weight of each leaf sample) of ice-cold Nanopure water were added to the tube, the tube was mixed using a vortex and kept on ice. Plant samples were then centrifuged at 4°C and 14, 000 rpm for 30 min. The supernatant was extracted from the sample (~900 µL) and ~500 µL was filtered through a PURADISC™ 25 mm 0.22 µm pore size disposable nylon syringe filter (GE Healthcare UK Ltd., Buckinghamshire, UK), into a new amber glass HPLC vial. The remaining supernatant was put in a new microcentrifuge tube and kept in -20°C for future use.

2.3.3 HPLC parameters

Plant samples for analysis of ureides were injected onto a Aminex® HPX-87H Organic Acid column (300 x 7.8 mm) (Bio Rad Laboratories, Hercules, CA, USA) using a 2.5 mM H_2SO_4 running phase, (pH ~3.0) on a 1200 Series Agilent high performance liquid chromatography (HPLC) system (Agilent Technologies Canada Inc., Mississauga, ON, Canada). The HPLC column uses the mechanisms of ion exclusion, ion exchange, ligand exchange, size exclusion, reversed phase, and normal phase partitioning all to separate out compounds of interest. The flow rate was set at 0.5 mL per min at 20.0°C for a total run-time of approximately 40 to 45 min. The compounds of interest were read at an absorbance of 191 nm with a 2 nm bandwidth using a diode array detector. Identification of compounds was based on retention time and absorbance spectrum compared to pure standards. Allantoin, uric acid, and allantoate were routinely detected at approximately 19 min, 21.5 min, and 24 min, respectively. The average injection volume was 20 µL, ranging from 10 to 50 µL. Data collection was performed using ChemStation software (Agilent Technologies). A standard curve was generated using pure compounds (Sigma Aldrich, Oakville, ON, Canada). For each compound, area under the peak was used to calculate the amount of each ureide. Standard curves had an R^2 value of 0.999, 0.997, and 0.999 for allantoin, uric acid, and allantoate, respectively.

2.3.4 Statistical analysis of ureide accumulation following water limitation

HPLC chromatograms were analyzed and peaks for allantoin, uric acid, and allantoate were noted and the area under each peak was used to calculate the amount of each ureide (in nmol) using a standard curve generated for each compound. Statistical analysis of the water-limitation experiment data was carried out using an analysis of variance for non-parametric data, comparing the variance of the median values of ranked data between the biological replicates to observe if there was any statistical difference between genotypes, treatments or days of water limitation with confidence interval or p-value <0.05 with the program IMB® SPSS® Statistics, version 21.

2.3.4.1 Initial data exploration

The data were not normally distributed as indicated by parametric analysis using ANOVA with two fixed factors (Inoculation x Water). The Levene's Test of Equality of the Error Variances, which tests the null hypothesis that the error variance of the dependent variable was equal across groups, was used. Highly significant p-values were obtained, which suggests that the variances of the data are not equal and that the ANOVA test should not be used to analyze the data. Graphs plotting the parametric observed x predicted x standard residual plots showed non-normally distributed data for much of the data, therefore, a decision was made to analyze the data by either parametric or non-parametric analyses. Since all of the data were not normally distributed under parametric analyses, non-parametric analyses were chosen because the same statistical analysis should be performed on all the data.

The non-parametric Mann Whitney U Test was employed to compare all the data for both genotypes separated by day and significant p-values were returned for less than half of the data (Appendix F). Overall, the tests revealed that there were some significant differences in ureides between the two genotypes, however this was not in all cases, therefore, the data were analyzed separately by genotype using non-parametric analyses.

A non-parametric analysis involves having ranked data and comparing the median values of the ranked data, not the mean values as in parametric analysis. The first analysis carried out was the Friedman Test comparing all data for the different treatments ('water' and 'fixing' treatments) separated by day for each cultivar. The Friedman Test is a non-parametric version of the repeated-measures ANOVA that is performed on ranked data.

2.3.4.2 Analysis of nitrogen fixation on ureide accumulation in water limited faba bean

To look into the effect of the treatments, water and fixation as well as the interaction between treatments on ureides for each genotype and for each day, a two-way ANOVA using the Friedman Test was used. The Total Mean Squares for each interaction were calculated using the Sum of Squares for all factors including error, divided by the additive degrees of freedom (df) for all factors. This calculated value was compared to a critical value for each interaction to see if it was less than or greater than the critical value from

the chi-squared distribution table. If the calculated value was greater than the critical value, the test resulted in a significant result, $p<0.05$.

CHAPTER 3. RESULTS

3.1 Quantification of total ureides in field grown faba bean

3.1.1 Quantification of ureide concentration at growth stage anthesis

There was no significant effect of genotype on ureide concentration ($p = 0.4822$) but a significant relationship existed between plant partition and ureide content ($p<0.0001$, Table 3.1). The latter relationship was analyzed using a series of contrasts (Table 3.2). A series of three contrasts between plant partitions; leaf, stem, and reproductive were used to observe which plant partition had the greatest effect on ureide concentration. The p-values were 0.4724 comparing leaf and reproductive parts, and $p<0.0001$ for comparing both leaf and stem and reproductive and stem partitions (Table 3.2). A bar plot of the average ureide concentration in plant partitions at growth stage anthesis shows significant differences between ureides in plant partitions, indicated by different letters (Fig. 3.1).

Table 3.1. ANOVA table showing the effects of genotype and plant partition on ureide concentration in faba bean using the mixed model for plants sampled at growth stage anthesis.

ANOVA				
	DF	denDF	F-value	P-value
Intercept	1	234	321.4943	<0.0001
Genotype	14	112	0.9761	0.4822
Plant Partition	2	234	1604.9892	<0.0001
Genotype x Plant Partition	28	234	0.4739	0.9897

Table 3.2. Results for the three contrasts for the fixed factor plant partition at anthesis.

Leaf	Reproductive	Stem	AIC	Value	Std. Error	DF	t-value	p-value
-1	1	0	35.96947	0.0102509	0.01424563	262	0.71958	0.4724
-1	0	1	35.96947	0.7180442	0.01407037	262	51.03235	0.0000
0	-1	1	35.96947	0.7077932	0.01424563	262	49.68495	0.0000

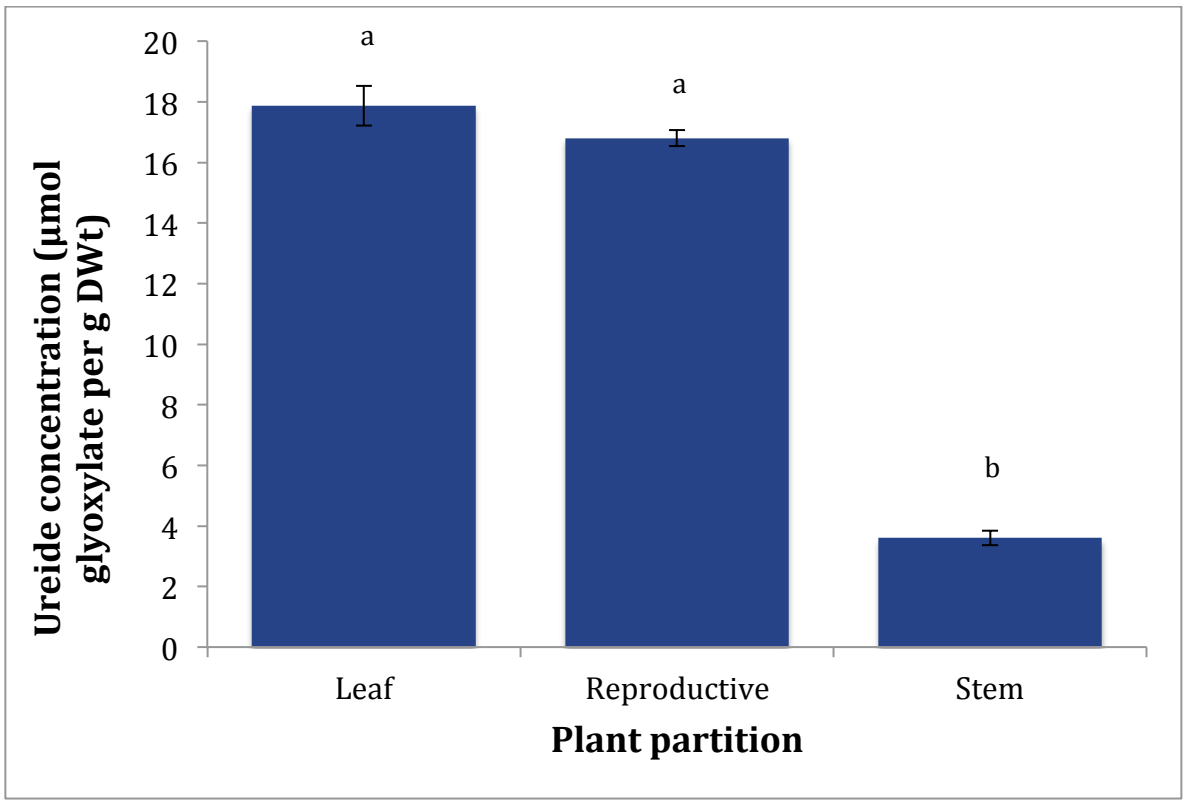

Figure 3.1. A bar plot of the average ureide concentration for each plant partition at growth stage anthesis. Mean ureide concentrations are 17.87, 16.80, and 3.60 for leaf, reproductive, and stem parts, respectively. Different letters above each bar indicate significant differences between plant partitions ($p<0.001$). Statistical analysis was performed on log-transformed data while this graph depicts the original un-transformed data.

3.1.2 Quantification of ureide concentration at growth stage mid-pod

The same process of data analyses was carried out on the growth stage, mid-pod, and the statistical results are as follows. At mid-pod, there was no significant effect of genotype on ureide concentration (p=0.8448), and there was no significant interaction between genotype and plant partition (p=0.9987); however, there was a significant difference in ureide concentration between plant partitions [p <0.0001, (Table 3.3)]. Contrasts of all three plant partitions returned significant values [p <0.0001, (Table 3.4)]. The average ureide concentration by plant partition had significant differences between all plant partitions sampled, indicated by different letters above each plot (Fig. 3.2).

Table 3.3. ANOVA table showing the effects of genotype and plant partition on faba bean log ureide content for the mixed model, Mixed 1, in plants sampled at growth stage mid-pod.

ANOVA				
	DF	denDF	F-value	P-value
Intercept	1	240	393.7114	<0.0001
Genotype	14	112	0.6187	0.8448
Plant Partition	2	240	386.9283	<0.0001
Genotype x Plant Partition	28	240	0.3697	0.9987

Table 3.4. Results for the three contrasts run on the fixed factor plant partition at growth stage mid-pod.

Leaf	Reproductive	Stem	AIC	Value	Std. Error	DF	t-value	p-value
-1	1	0	515.6478	0.129683	0.02653614	268	4.887033	0.0000
-1	0	1	515.6478	0.7139969	0.02653614	268	26.90658	0.0000
0	-1	1	515.6478	0.5843139	0.02653614	268	22.01955	0.0000

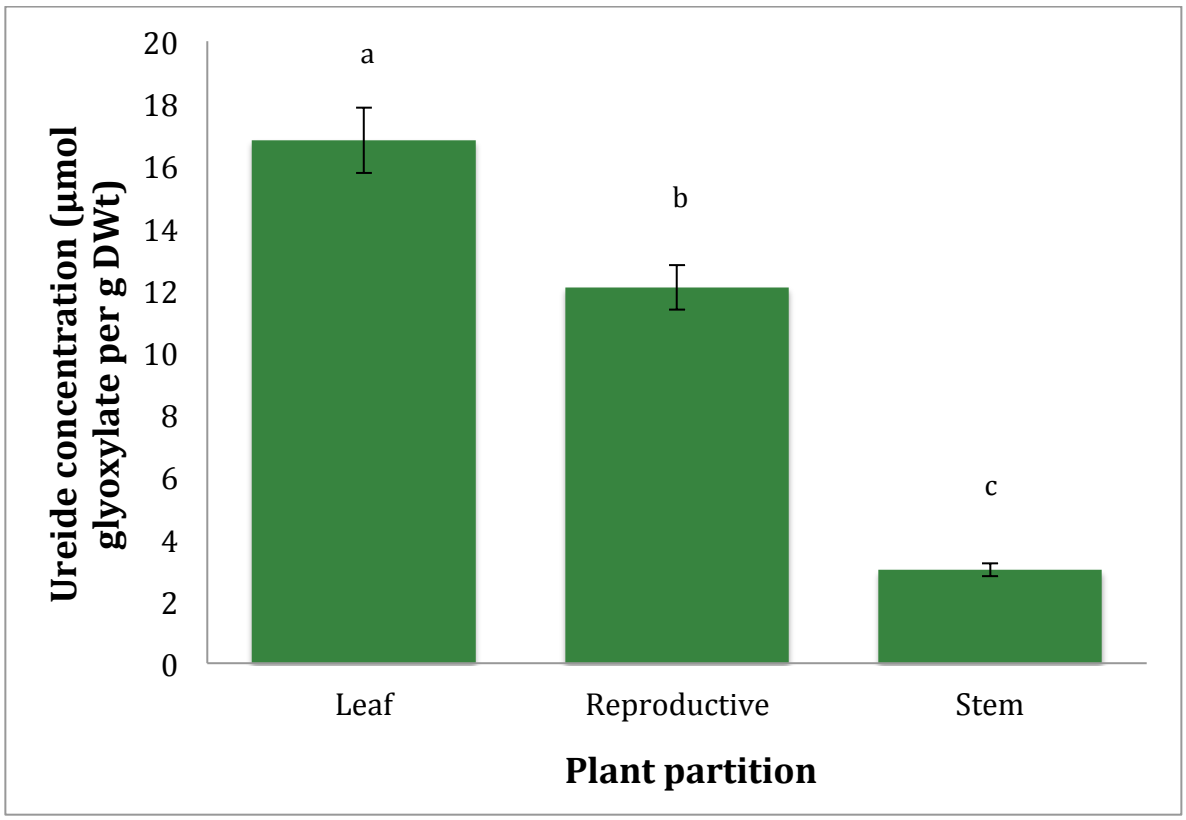

Figure 3.2. A bar plot of the average ureide concentration for each plant partition at growth stage mid-pod fill. Mean ureide concentrations are 16.81, 12.08, and 3.00 for leaf, reproductive, and stem partitions, respectively. Different letters above each bar indicate significant differences between plant partitions ($p<0.001$). Statistical analysis was performed on log-transformed data while this graph depicts the original un-transformed data.

3.1.3 Quantification of ureide concentration at growth stage physiological maturity

Statistical analysis at physiological maturity showed similar results. Again, there was no significant effect of genotype on ureide concentration ($p=0.5958$), and no significant interaction between genotype and plant partition ($p=0.7573$), and again, there was a significant difference in ureide concentration between plant partitions ($p<0.0001$, Table 3.5). The same parameters for model selection were used as previously described. Again, contrasts were run on all four plant partitions (leaf, stem, reproductive (non-seed), and seed) returning significant values ($p<0.001$) for all contrasts (Table 3.6). A significant difference among all plant partitions and ureide concentration is shown in the bar plot

below, where significant differences are indicated by different letters above each bar plot (Fig. 3.3).

Table 3.5. ANOVA table showing the effects of genotype and plant partition on log ureide concentration in faba bean for the mixed model, Mixed 1, in plants sampled at physiological maturity.

ANOVA				
	DF	denDF	F-value	P-value
Intercept	1	357	589.2527	<0.0001
Genotype	14	112	0.8671	0.5958
Plant Partition	3	357	313.8203	<0.0001
Genotype x Plant Partition	42	357	0.8357	0.7573

Table 3.6. Contrast results for the fixed effect plant partition, at growth stage physiological maturity.

Leaf	Reproductive	Seed	Stem	AIC	Value	Std. Error	DF	t-value	p-value
-1	1	0	0	604.7231	0.4133231	0.02477698	399	16.68174	0.000
-1	0	0	1	604.7231	0.7125051	0.02477698	399	28.75673	0.000
-1	0	1	0	604.7231	0.1486116	0.02491999	399	5.96355	0.000
0	-1	0	1	604.7231	0.2991820	0.02477698	399	12.07499	0.000
0	-1	1	0	604.7231	-0.2647115	0.02491999	399	-10.62245	0.000

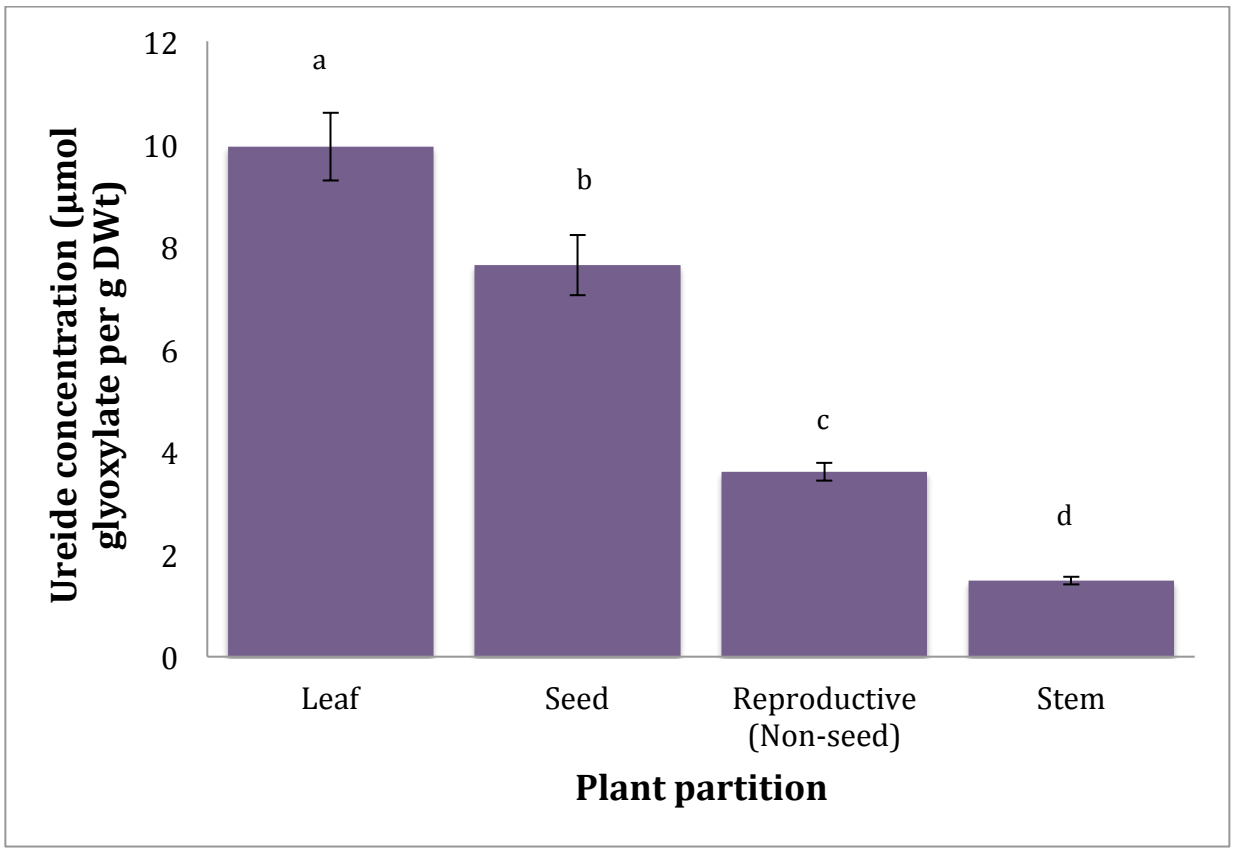

Figure 3.3. A bar plot of the average ureide concentration for each plant partition at growth stage physiological maturity. Mean ureide concentrations are 9.95, 7.63, 3.60, and 1.48 for leaf, seed, reproductive (non-seed), and stem partitions, respectively. Different letters above each bar indicate significant differences between plant partitions ($p<0.001$). Statistical analysis was performed on log-transformed data while this graph depicts the original un-transformed data.

3.1.4 Quantification of ureide concentrations in nodule field samples

A subset of the original samples was analyzed for the amount of total ureide glyoxylate equivalents determined as µmol ureide per g of dry weight tissue. The results showed that ureides were present in the root nodules in low quantities. The data were normally distributed so no transformation of the data was carried out (Appendix F). Statistical analysis showed that total nodule ureides differed significantly between time points sampled at anthesis, mid-pod fill, and physiological maturity based on the ANOVA results, $p=0.0019$ (Table 3.7). From statements contrast, a significant difference in ureide concentration was found between anthesis and physiological maturity time points ($p=0.0048$), a greater significant difference in ureide concentration between mid-pod and

physiological maturity was observed (p=0.0009), but no significant difference between anthesis and mid pod was determined (Table 3.8). A bar plot of the data shows the distribution of ureides at each sampling time with significant differences between sampling times indicated by different letters (Fig. 3.4).

Table 3.7. ANOVA table showing the significant p-value (0.0019) for the fixed factor time for ureides analyzed in nodules.

ANOVA				
	DF	denDF	F-value	p-value
Intercept	1	18	298.49196	<0.0001
Time	2	18	9.09551	0.0019

Table 3.8. Results from the three contrasts for ureides found at each growth stage.

Anthesis	Mid-Pod	Physio-logical Maturity	AIC	Value	Std. Error	DF	t-value	p-value
-1	1	0	100.9037	0.096650	0.1962052	18	0.492594	0.6283
-1	0	1	100.9037	-0.621576	0.1932182	18	-3.216963	0.0048
0	-1	1	100.9037	-0.718225	0.1816180	18	-3.954593	0.0009

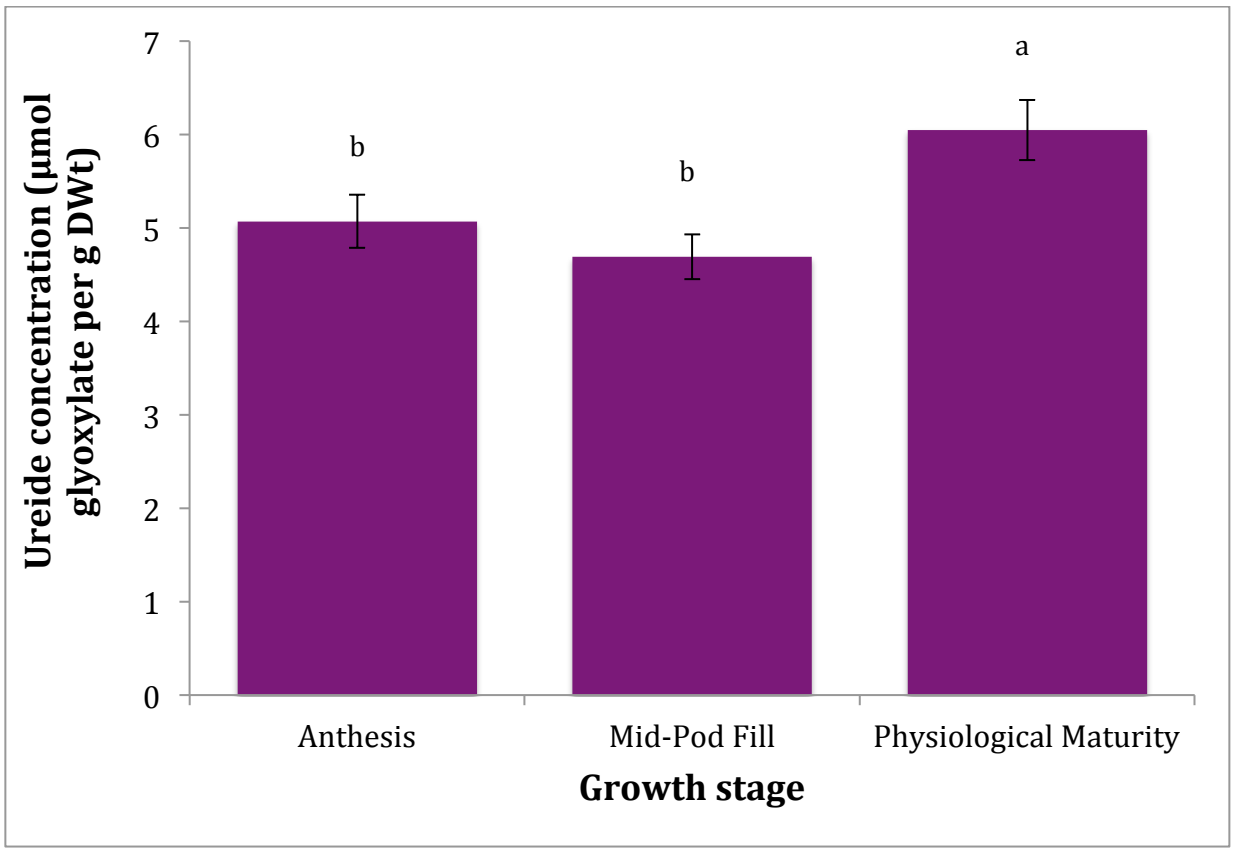

Figure 3.4. The mean ureide concentration in nodules by growth stage. Mean values for each sampling time are 5.07, 4.69, and 6.05 for anthesis, mid-pod fill and physiological maturity, respectively. There was a significant difference in ureide content between nodule concentrations at anthesis and physiological maturity and mid-pod and physiological maturity ($p<0.01$ and $p<0.001$, respectively). Statistical analysis was performed on log-transformed data while this graph depicts the original un-transformed data.

Overall, the faba bean field data showed a high amount of ureides found in the leaves, followed by reproductive parts, nodules, and lastly stem tissue. The amount of ureides decreased over the sampling periods of anthesis, mid-pod fill and physiological maturity for all partitions sampled except nodules, which showed an increase in ureides towards the end of the plant life cycle, at physiological maturity. There were no significant differences in ureide concentrations between the fifteen different genotypes tested; however, there were significant differences in ureide concentrations in the different plant partitions tested for the various sampling times. The most significant change, or decrease in ureide concentration was observed at the end of the growing season for all plant partitions, and a significant positive change occurred in nodules at physiological maturity.

3.2 Sequences of allantoinase and allantoate amidohydrolase genes involved in ureide catabolism in faba bean

In order to generate tools to study ureide metabolism in faba bean at the molecular level, I attempted to clone two genes involved in ureide catabolism. The genes selected code for two of the best-characterized ureide metabolic enzymes in legumes (Werner et al., 2008), one that catabolizes allantoin into allantoate, allantoinase (*ALN*), and the subsequent enzyme in the metabolic pathway that converts allantoate into ureideoglycine, ammonium, and CO_2, allantoate amidohydrolase (*AAH*).

3.2.1. *Vicia faba* allantoinase (*VfALN*) sequence amplification

A 234 bp fragment of the faba bean allantoinase gene was amplified from 8 day-old faba bean hypocotyl cDNA, cloned and sequenced (Fig. 3.5), and a BLASTN search of the non-redundant nucleotide database (NCBI; www.ncbi.nlm.gov) identified the sequences sharing the greatest degree of nucleotide similarity as predicted allantoinase genes from *Medicago truncatula* XM_003593474.1 (91% nucleotide sequence identity), *Robinia pseudoacacia* AY466437.1 (black locust, 90% identity), *Cicer arietinum* XM_0044485692.1 (88%), 86 to 88% identity with several *Glycine max* XM_003543006.2 sequences and 86% identity with *Phaseolus vulgaris* Q277455.1. A BLASTX search also identified legume allantoinases as the highest matches, sharing 94% of amino acid residues to ALN in black locust AAR29343.1, 94% identical to chickpea ALN XP_004485749.1, 92% identical to ALN in *M. truncatula* XP_003593520.1, 87 to 90% identical to soybean ALN XP_003543054.1, and 87% identical to ALN in common bean XP_007148177.1.

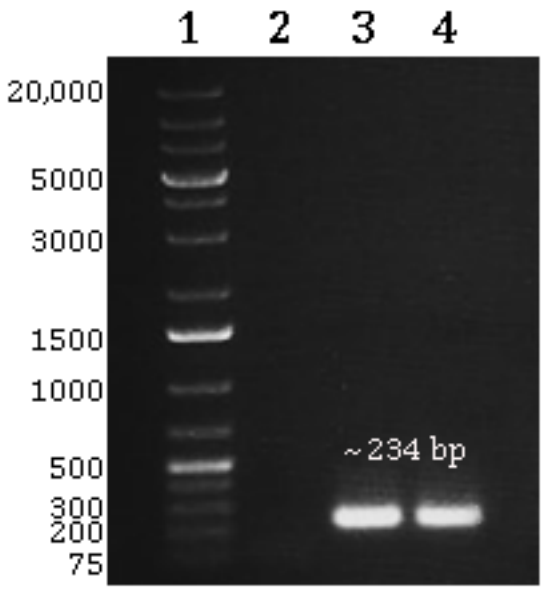

Figure 3.5. PCR amplification of *VfALN* sequence showing a 234 bp fragment visualized on an agarose gel. Lane 1: DNA ladder, lane 2: negative control, lane 3: amplification product from Fatima hypocotyl cDNA, lane 4: amplification product from NPZ4-7540 hypocotyl cDNA.

3.2.2 *Vicia faba* allantoate amidohydrolase (*VfAAH*) sequence amplification

A 529 bp fragment was amplified from faba bean seedling cDNA, cloned and sequenced (Fig. 3.6) and the sequencing results were used to search the non-redundant nucleotide database with a BLASTN search (www.ncbi.nlm.gov). The sequence from a 529 bp fragment amplified from faba bean cDNA resulted in a shared 97 to 99% identity with three *Glycine max AAH* genes XM_006586886, 94% identity with a putative *Phaseolus vulgaris AAH* gene EF650088.2, an 89% match to a *Lotus japonicas* (wild legume species) clone BT137626.1, an 88% identical match for the *AAH* gene in *Medicago truncatula* XM_003594803.1, and an 87% identical nucleotide sequence to a *Cicer arientinum* predicted *AAH* gene XM_004487852.1, strongly suggesting that the primers used in this study amplified faba bean *AAH*. A BLASTX search was performed and amino acid sequences annotated as *AAH* from the following legume species were returned as the top results: four hits to soybean CAO78893.1 (98 to 99% amino acid identity), common bean ABR57240.2 (97%), chickpea XP_004487909.1 (89%), and *M. truncatula* XP_003594851.1 (86%).

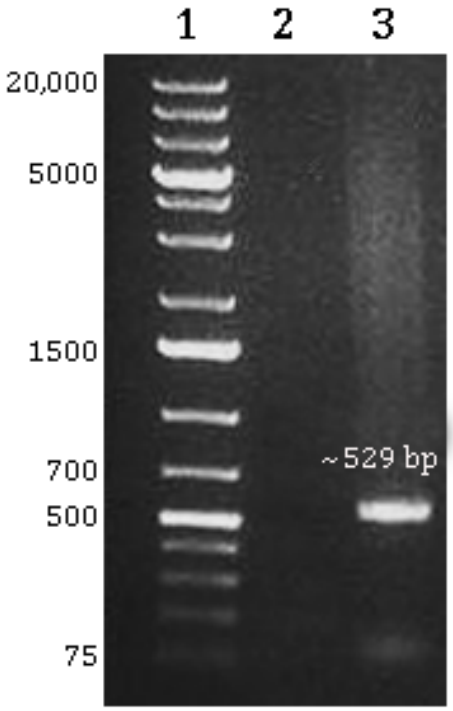

Figure 3.6. PCR amplification of a 529 bp fragment from *VfAAH* visualized on an agarose gel. Lane 1: DNA ladder, lane 2: negative control, lane 3: amplification product from Fatima leaf cDNA.

3.2.3 *Vicia faba* actin housekeeping reference gene amplification

PCR primers designed to amplify a 474 bp fragment, successfully amplified *VfACT* from 42 day-old faba bean leaf cDNA (Fig. 3.7). A BLASTN search of the non-redundant nucleotide sequence database was performed for the housekeeping reference gene and a 455 bp region was identified as 86% identical to the faba bean EST from which the primers were designed. The BLASTN search also returned highly similar nucleotide sequences in *Pisum sativum* (87% similar HQ231775), *M. truncatula* (84% similar # BT141409), *P. vulgaris* (82% similar XM_007139153), and to *Lotus japonicas* (82% similar AK339288).

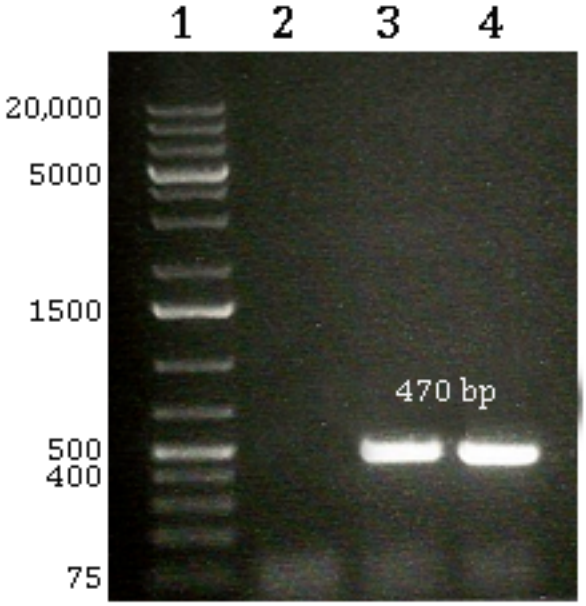

Figure 3.7. The amplification of a 470 bp PCR product from the *VfACT* gene, visualized by gel electrophoresis. Lane 1: DNA ladder, lane 2: negative control, lane 3: amplification product from Fatima leaf cDNA, lane 4: amplification product from NPZ4-7540 leaf cDNA.

3.2.4 Amplification of faba bean *ALN, AAH* and *Actin* transcripts

ALN was amplified from hypocotyl tissue and *AAH* was amplified from leaf tissue. Actin primers amplified a band of the expected size from faba bean leaf tissue. Based on these preliminary results it appeared that the primers designed could be used to assess changes in *ALN* and *AAH* gene expression.

3.3 Ureide accumulation following water limitation in faba bean

Faba bean plants from two genotypes (Snowbird and NPZ4-7540) were subjected to four different treatments (1. 80% SWC, inoculated, 2. 20% SWC, inoculated, 3. 80% SWC, non-inoculated, 4. 20% SWC, non-inoculated) in a water limitation experiment and were analyzed for ureide concentration over a 10-day water limitation period using HPLC analysis (Fig. 3.8 & 3.9). The results of these analyses are shown in the following sections.

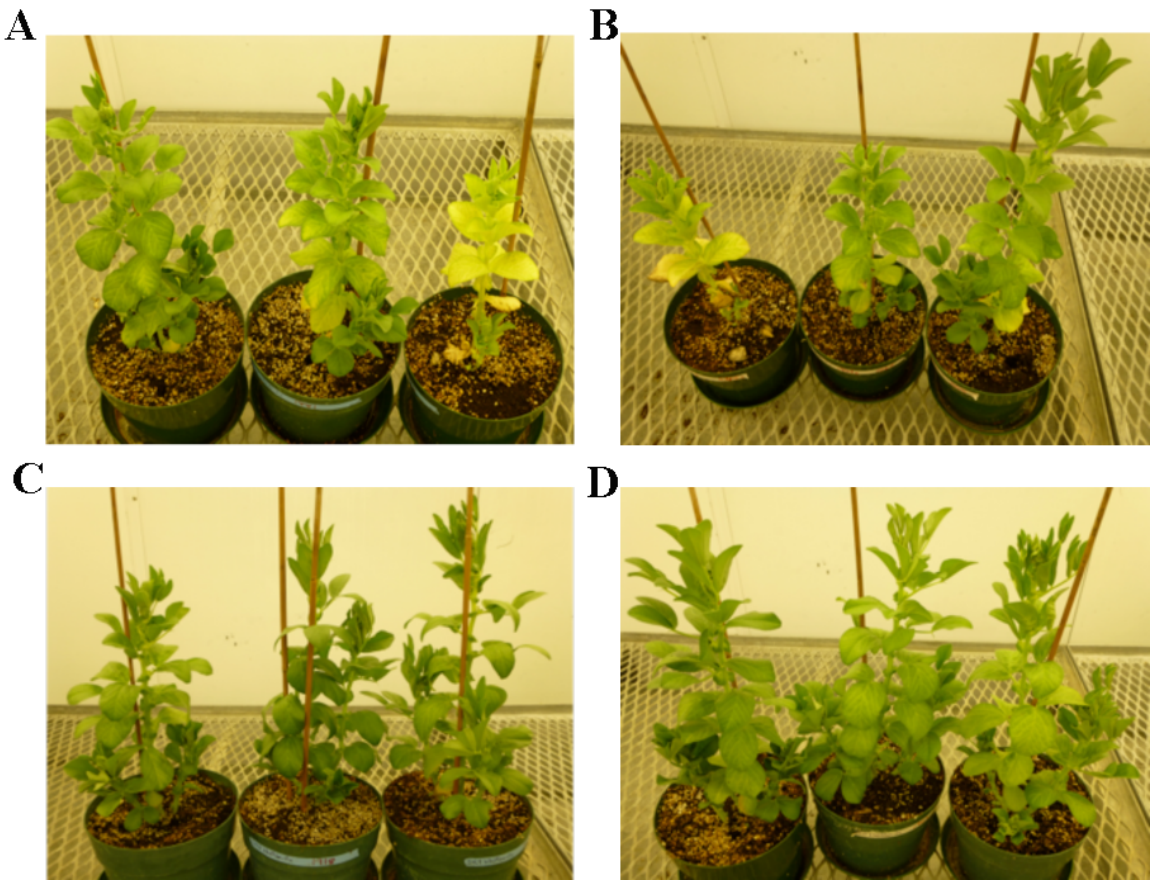

Figure 3.8. Representative faba bean plants ('Snowbird' genotype) from the water limitation experiment, grown in a growth chamber. A.) well-watered (80% SWC) and inoculated, B.) low-watered (20% SWC) and inoculated, C.) well-watered (80% SWC) and uninoculated, D.) low-watered (20% SWC) and uninoculated.

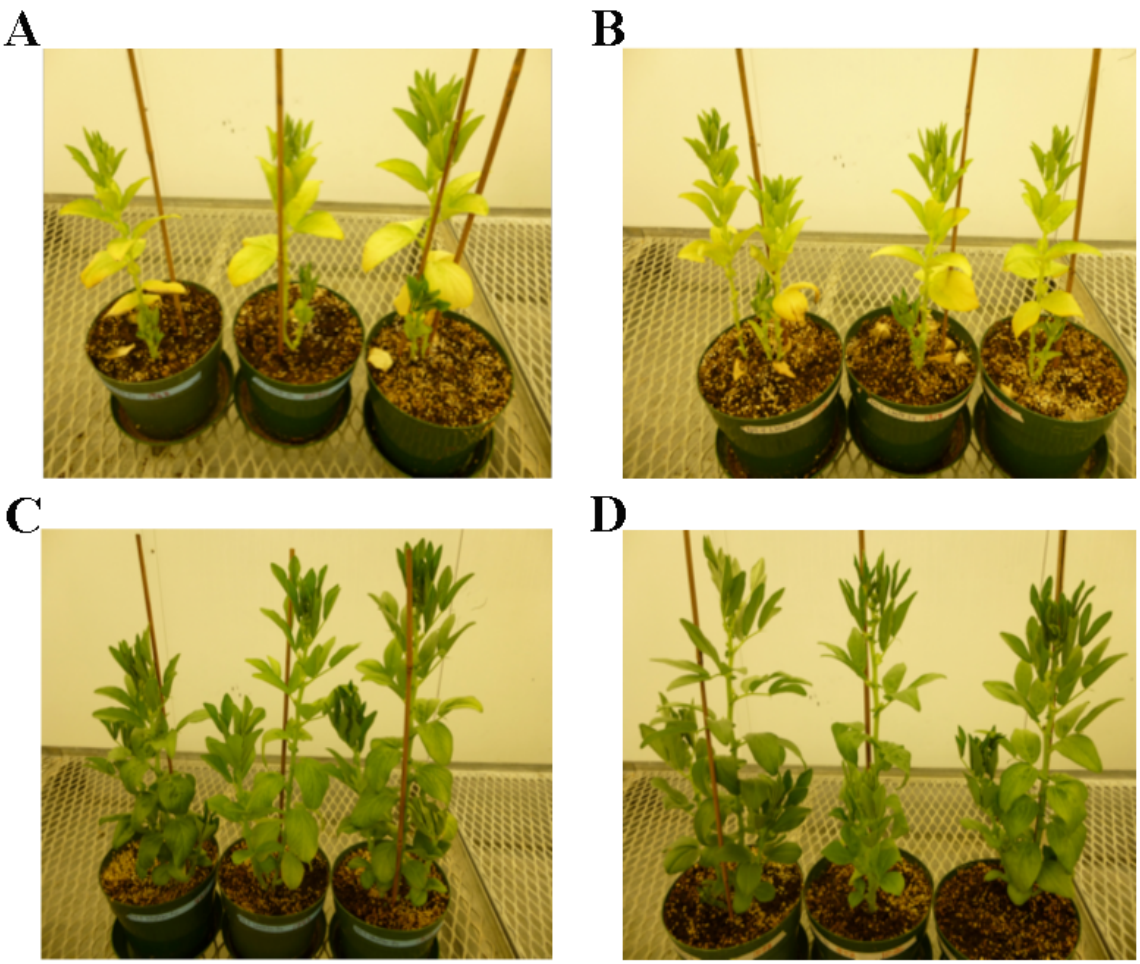

Figure 3.9. Representative faba bean plants ('NPZ4-7540' genotype) from the water limitation experiment, grown in a growth chamber. A.) well-watered (80% SWC) and inoculated, B.) low-watered (20% SWC) and inoculated, C.) well-watered (80% SWC) and uninoculated, D.) low-watered (20% SWC) and uninoculated.

3.3.1 Quantification of ureide accumulation following water limitation

In analyzing the amount of ureides accumulated in each genotype of faba bean according to the day of water limitation, the following results were obtained. In Snowbird, allantoin accumulated over the 10-day period especially in days 8 and 10. Using a Post-hoc test, a multiple comparison method similar to the Tukey test for parametric ANOVAs, which is based on the sum of ranks, different letters show ureide values that are significantly different at a threshold of $p=0.05$. Significant differences in ureides were noted in Snowbird for allantoin at days 8 and 10 ($p<0.05$) (Fig. 3.10) showing a significant increase in allantoin over the water limitation period. Snowbird showed no significant

differences in uric acid (Fig. 3.11) but a declining trend in uric acid over the treatment period could be seen in the data. The ureide allantoate also had significant differences between days 8 and 10 (Fig. 3.12), with significant accumulation over the treatment of water limitation.

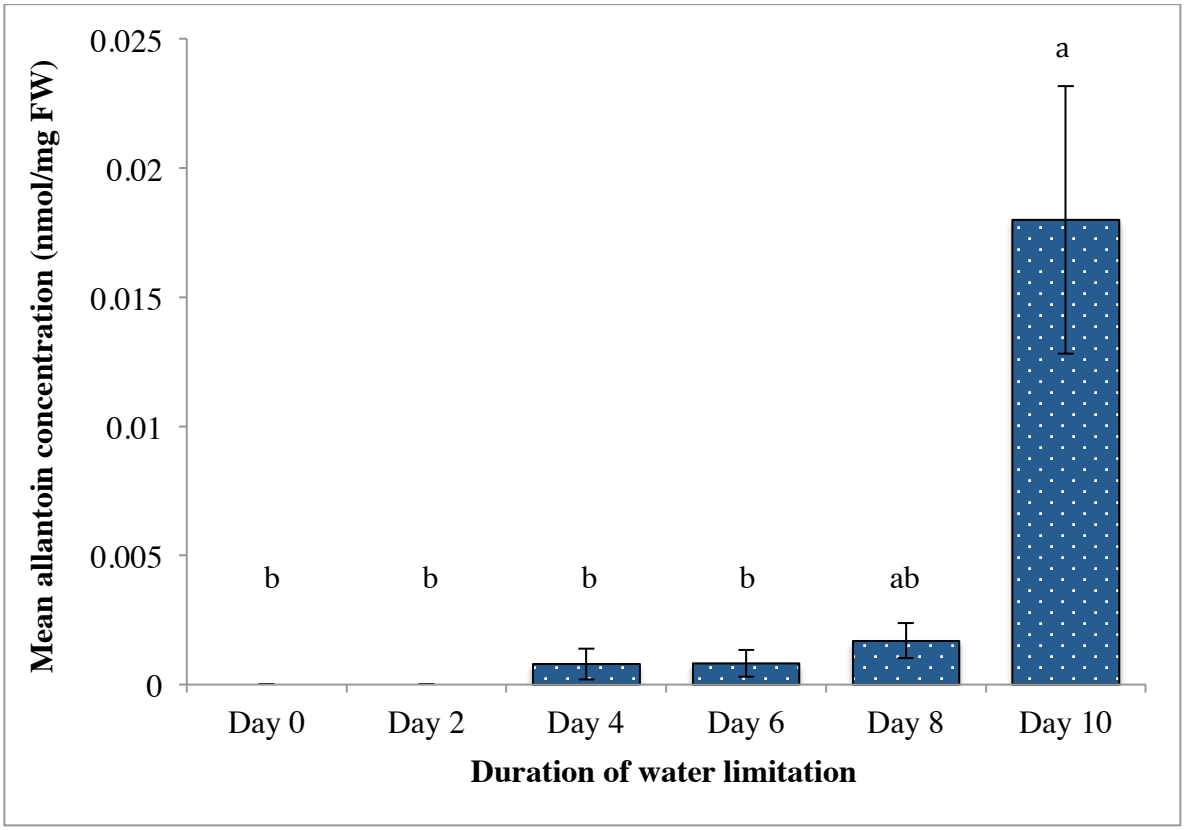

Figure 3.10. Comparison of the mean allantoin concentration in Snowbird plants over a 10-day water limitation period. Different letters above each day denotes significant differences, $p<0.05$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

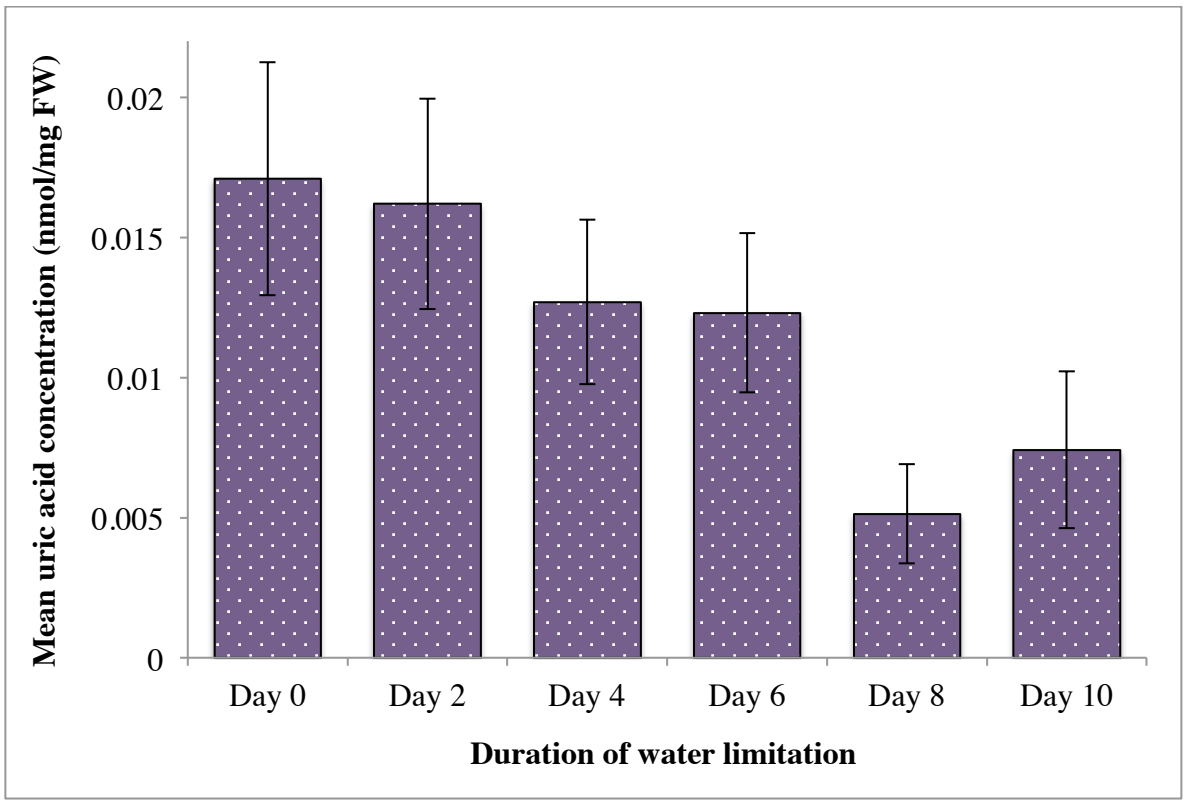

Figure 3.11. Comparison of mean uric acid concentration in Snowbird plants during a 10-day water limitation period. No significant difference in uric acid concentration was observed by day. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

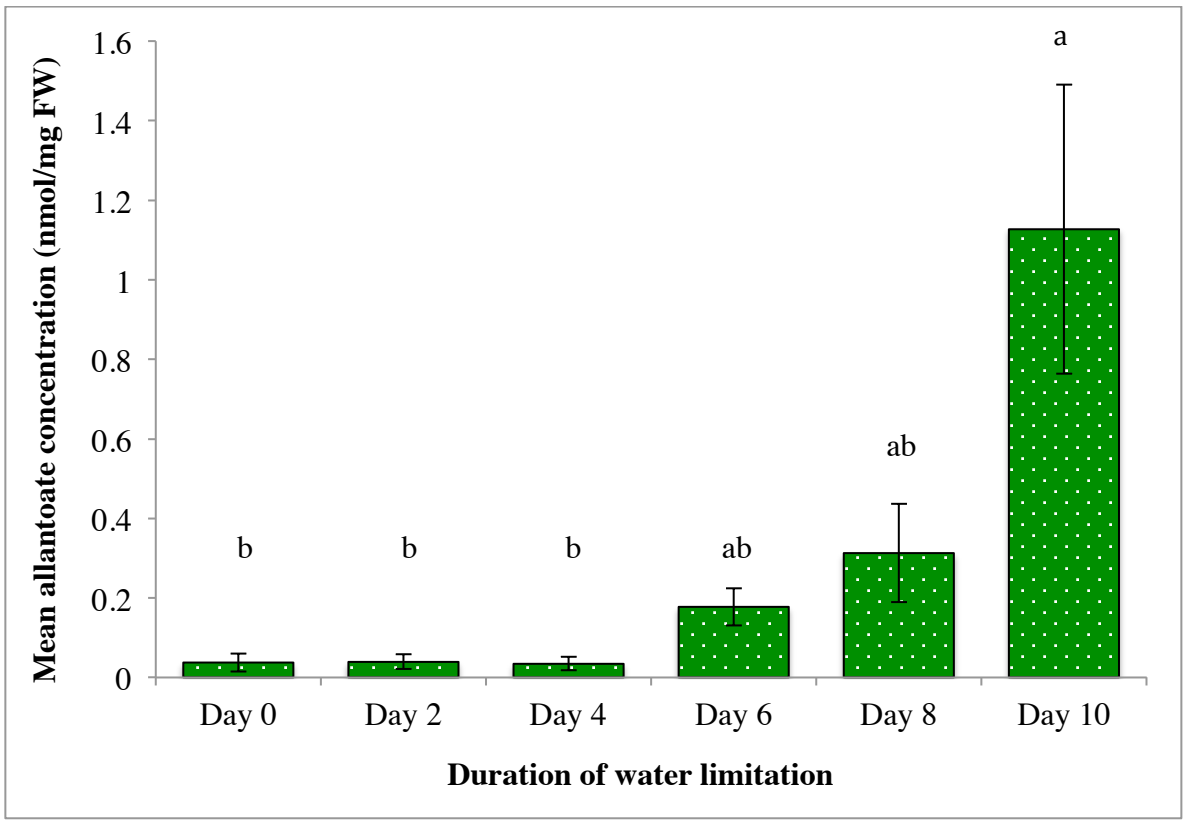

Figure 3.12. Comparison of mean allantoate concentration in Snowbird genotype over a 10-day water limitation period. Significant differences in allantoate by days indicated by different letters above each column, $p<0.05$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

For the cultivar NPZ4-7540, none of the three ureides showed significant differences over the treatment period. Allantoin showed no significant differences in ureides over the entire 10-day period (Fig. 3.13), however a general trend can be clearly noted with increasing allantoin over the 10-day period, with highest accumulation at day 10. Both uric acid and allantoate had no significant differences during the period of water limitation (Fig. 3.14 & 3.15). There are no visible trends in the data for both uric acid and allantoate for this genotype as the data are too variable.

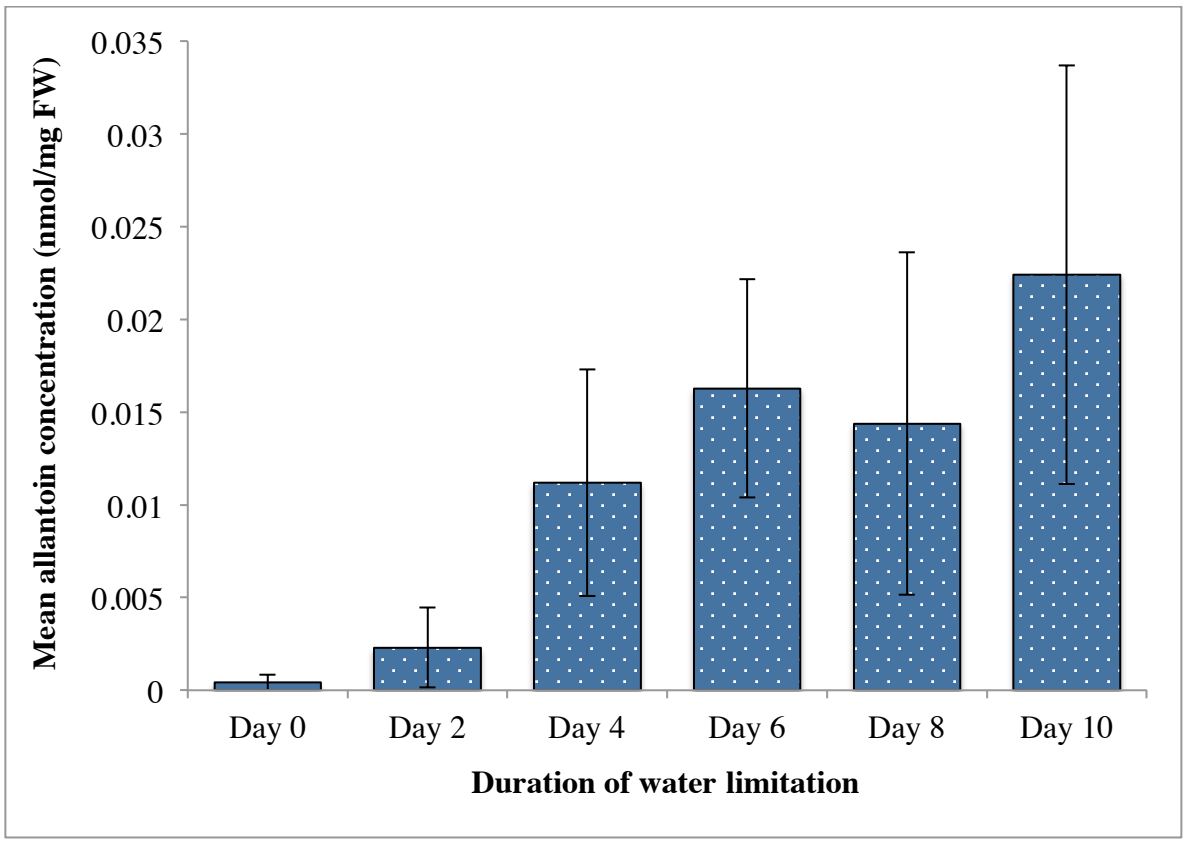

Figure 3.13. Comparison of mean allantoin concentration in NPZ4-7540 plants during a 10-day water limitation period. No significant differences in allantoin concentration were observed by day. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

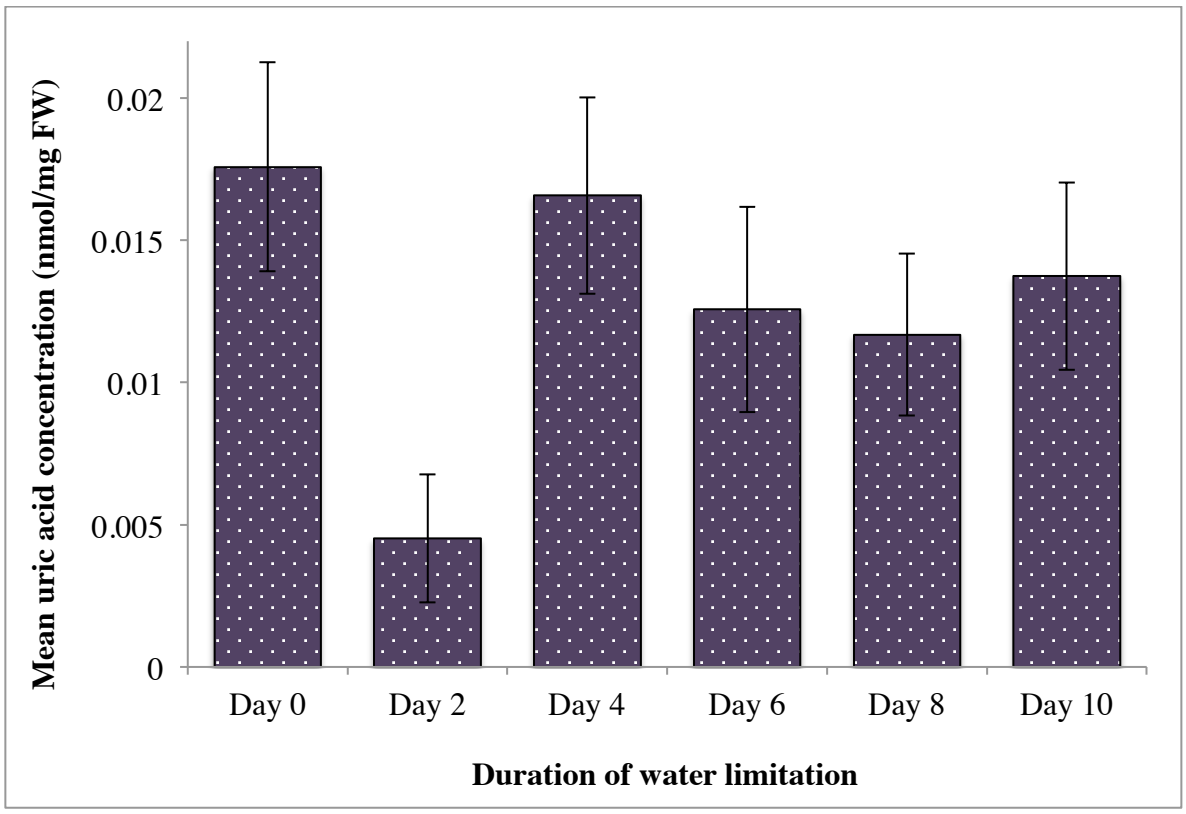

Figure 3.14. The mean concentration of uric acid in NPZ4-7540 plants during a 10-day water limitation period. There were no significant differences in uric acid by day and no visible trends in the data. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

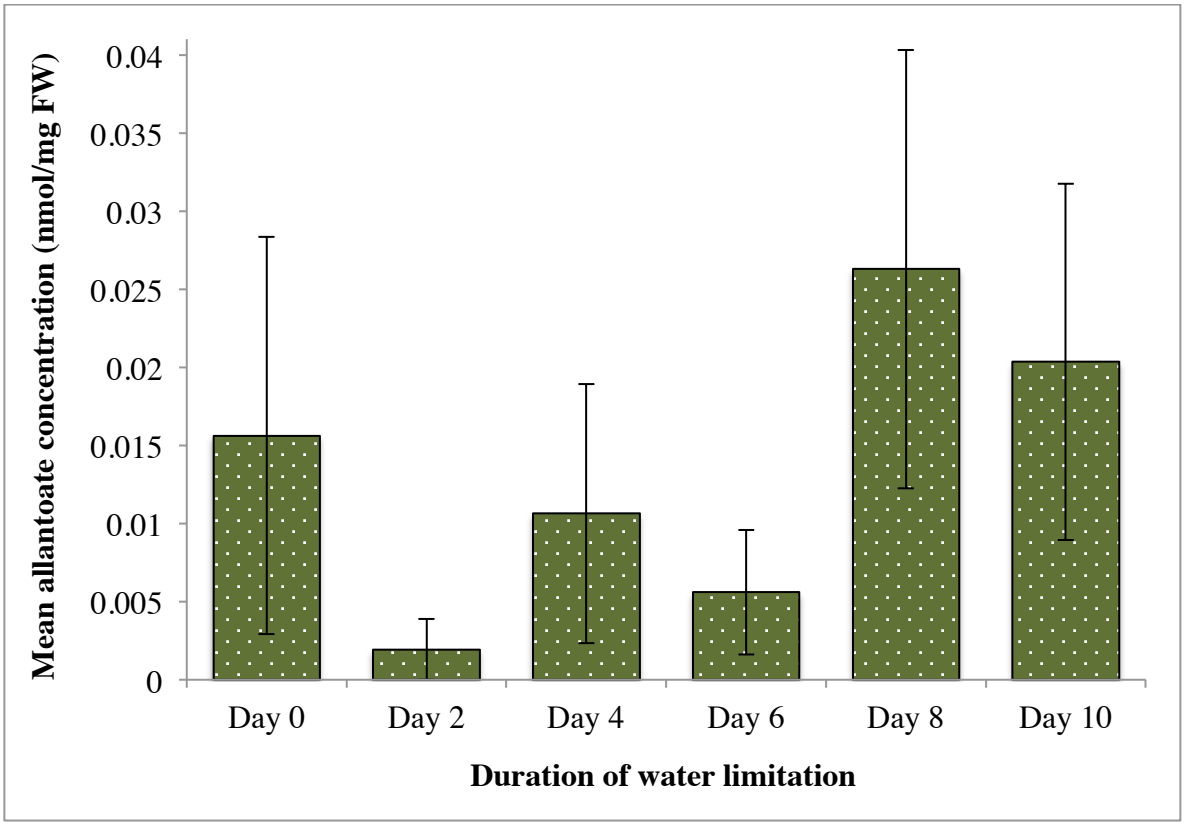

Figure 3.15. The mean allantoate concentration during a 10-day water limitation period in NPZ4-7540 plants. There were no significant differences or trends visible in allantoate by day. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=12). Error bars represent standard error of the mean.

3.3.2 Effects of inoculation and water treatment on ureide accumulation in water limited faba bean

In the genotype Snowbird, there was a significant effect of 'inoculation' on allantoin accumulation at day 8, $0.025<p<0.05$ (Fig. 3.16). There was also a nearly significant effect of 'inoculation' on allantoin at day 6 based on the calculated critical value. As seen in Fig. 3.16, the inoculated plants had significantly more allantoin than the non-inoculated plants. The data is showing that over the 10-day water limitation period, the plants with the most allantoin were the inoculated plants overall, with no effect of water treatment on the amount of allantoin accumulated.

For uric acid in Snowbird, there was a significant effect of 'water' on the amount of ureides present at day 8 (Fig. 3.17). There was significantly more uric acid present in the

low-watered vs. well-watered plants, $0.025<p<0.05$. But overall, there were no visible trends in the uric acid data, where most days, the uric acid concentration was roughly equal among treatment groups, however at days 0, 8, and 10 uric acid was more variable with no clear treatment having more or less ureides.

No significant effects due to 'inoculation' or 'water' treatment were noted for allantoate in Snowbird (Fig. 3.18). However, the data showed a slight trend of greater accumulation of allantoate in the well-watered inoculated and low-watered non-inoculated plants in the last three days of the experiment.

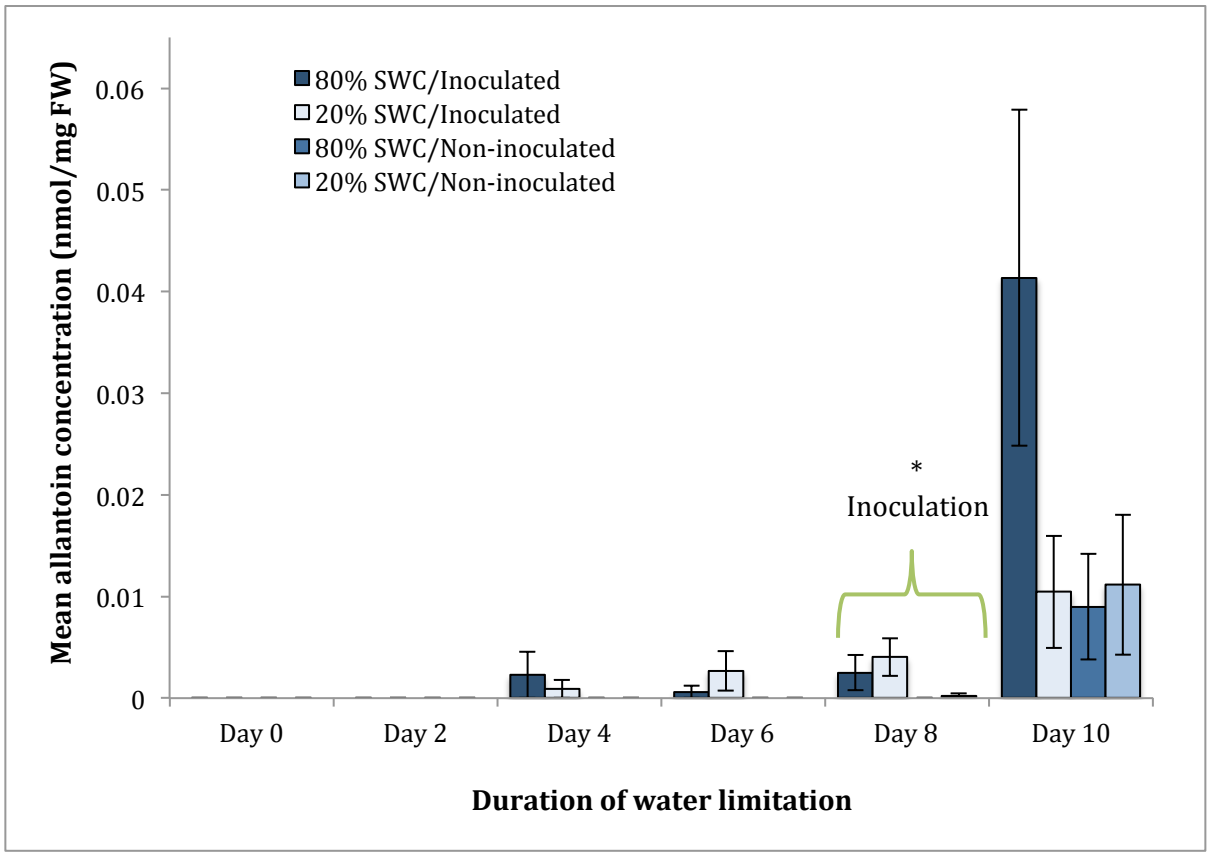

Figure 3.16. Mean allantoin concentration in Snowbird plants according to treatment type over a 10-day water limitation period. There was a significant effect of inoculation on allantoin at day 8, $0.025<p<0.05$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

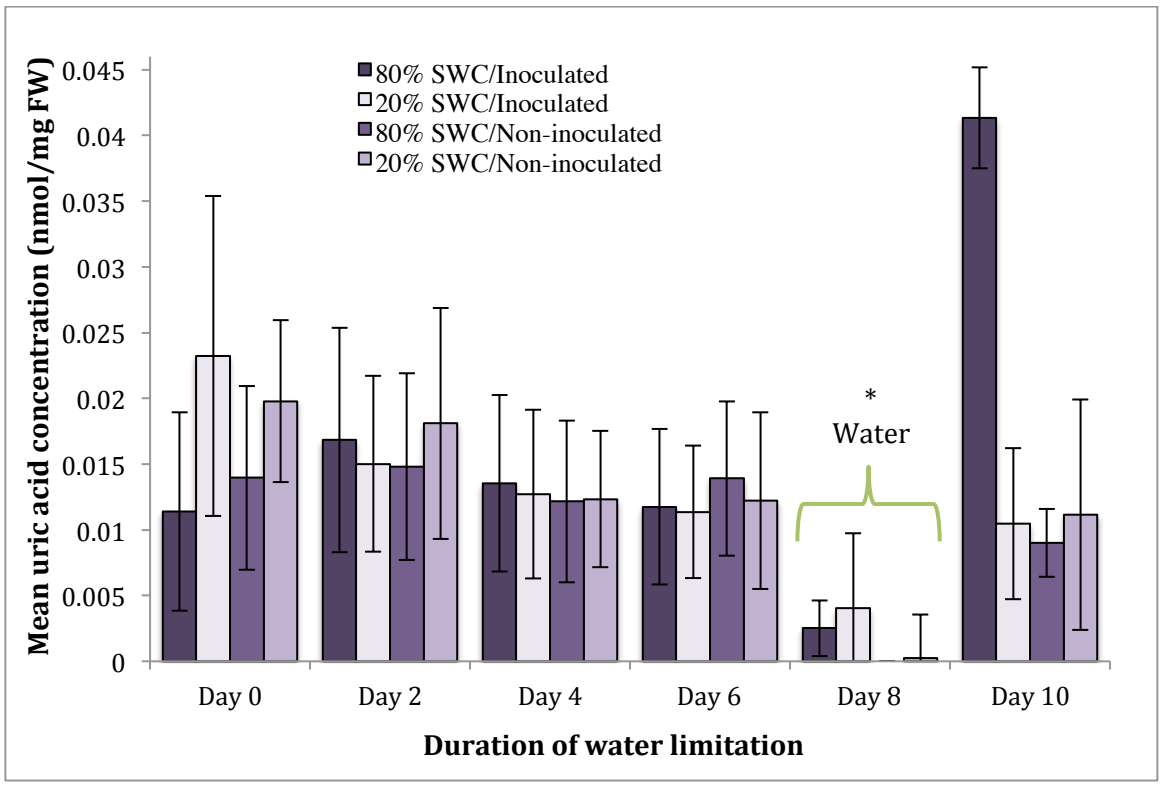

Figure 3.17. Mean uric acid concentration in Snowbird plants according to treatment type over a 10-day water limitation period. There was a significant effect of water treatment on uric acid concentration at day 8, $0.025<p<0.05$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

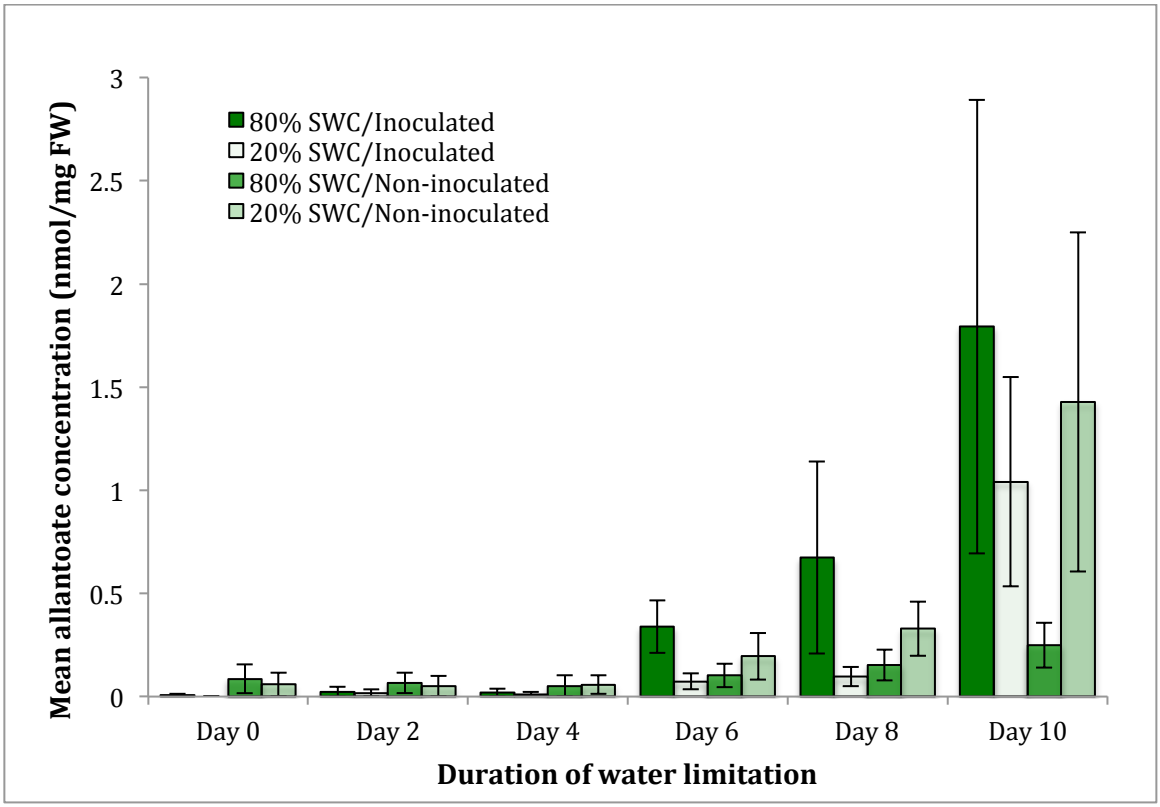

Figure 3.18. Mean allantoate concentration in Snowbird plants according to treatment type over a 10-day water limitation period. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

For the cultivar NPZ4-7540, the amount of allantoin was significantly affected by the factor 'inoculation' at days 8 and 10, $0.025<p<0.05$, respectively (Fig. 3.19). There was significantly more allantoin present in the inoculated versus uninoculated plants at day 8 and day 10. However, this trend was not evident for the other days of the experiment.

For uric acid, their concentration was significantly affected by 'inoculation', 'water', and the interaction between the two at day 2 of the experiment, $0.01<p<0.02$ (Fig. 3.20). There was significantly more uric acid present in the well-watered compared to low-watered plants, and significantly more uric acid in the uninoculated versus uninoculated plants. There was also an interaction between the two, where a well-watered treatment resulted in significantly more uric acid and with inoculation involved, uric acid was significantly accumulated in the well-watered non-inoculated plants compared to low-

watered inoculated plants. One trend noticeable from the uric acid data shows that the treatment resulting in the highest amount of uric acid overall was found in the well-watered uninoculated plants.

No effect of 'inoculation' or 'water' was noted in NPZ4-7540 for the ureide allantoate (Fig. 3.21), as the data was quite variable. The highest accumulation of allantoate was noted in the low-watered inoculated treatment over the course of the experiment.

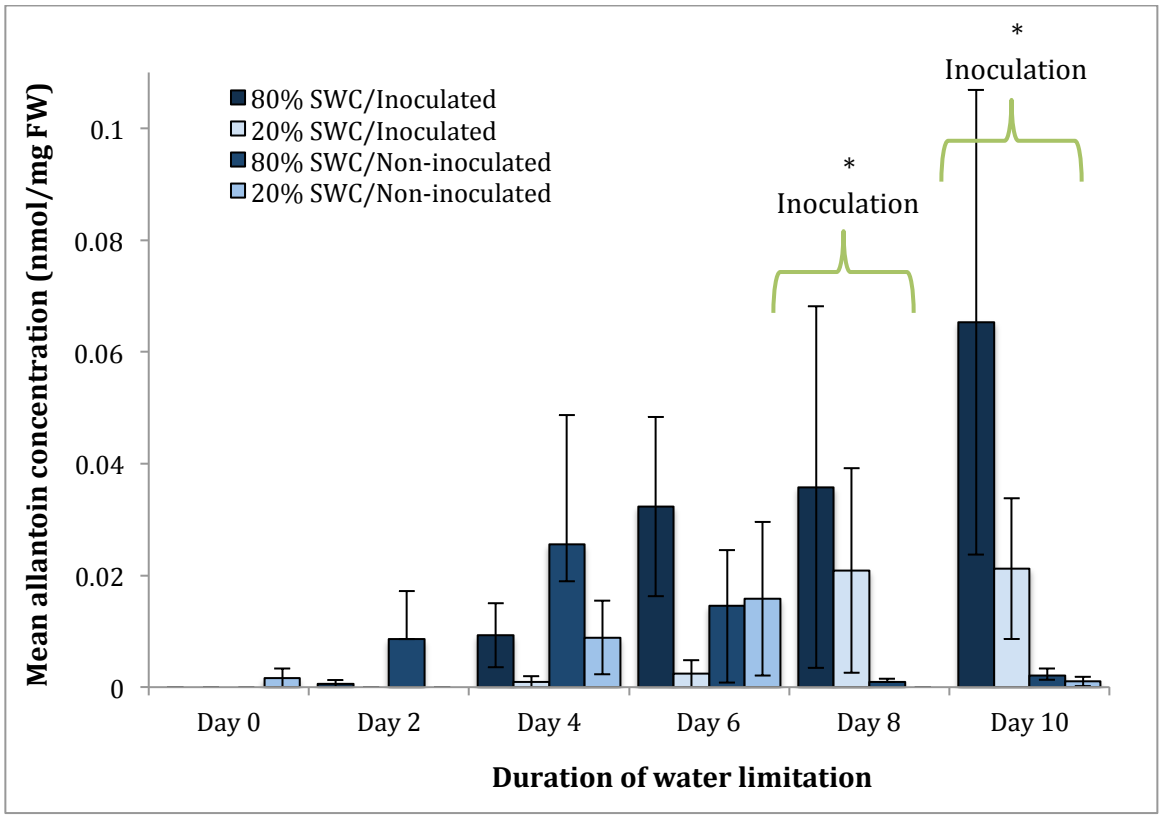

Figure 3.19. Mean allantoin concentration in NPZ4-7540 plants for the four treatment types during a 10-day water limitation period. Allantoin concentration significantly differs by 'inoculation' at days 8 & 10, $0.025<p<0.05$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

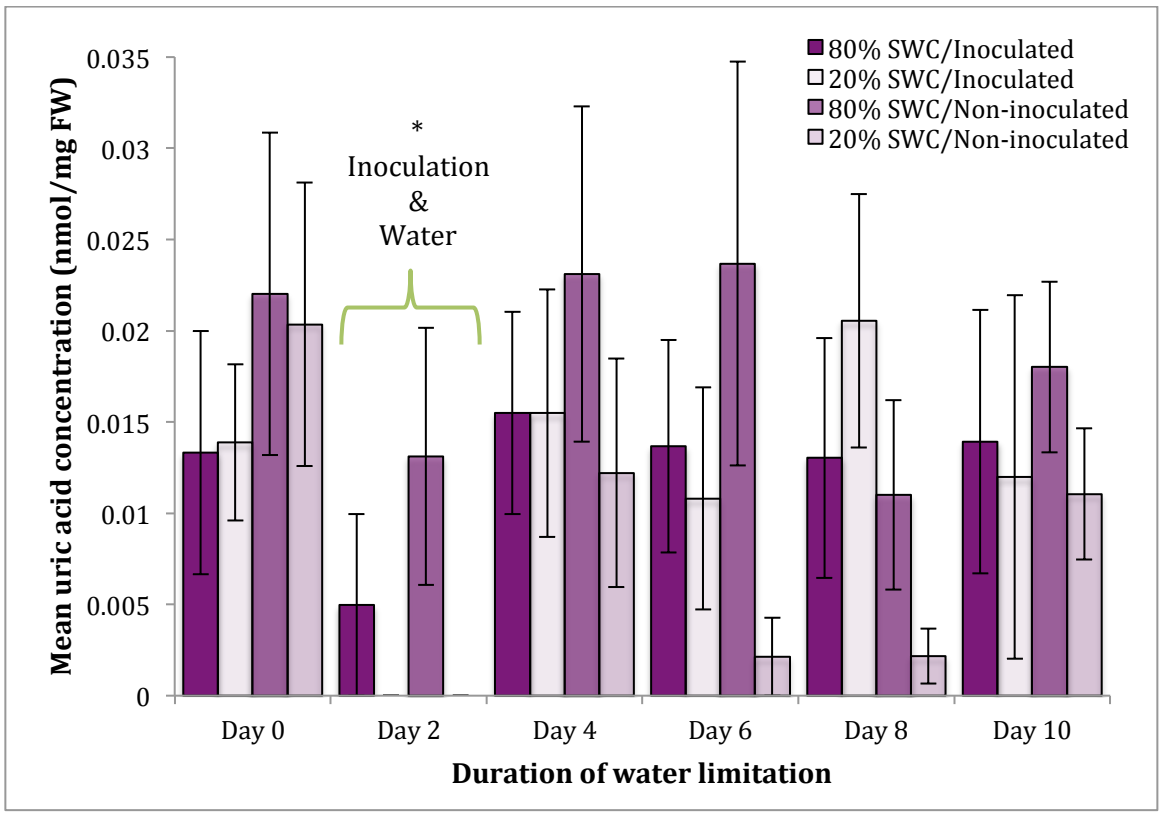

Figure 3.20. Mean uric acid concentration in NPZ4-7540 plants for the four treatment types during a 10-day water limitation period. Uric acid concentration significantly differs by 'inoculation' and 'water' treatment at day 2, $0.01<p<0.02$. There was also a significant interaction of 'inoculation' and 'water' on uric acid concentration at day 2, $0.01<p<0.02$. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

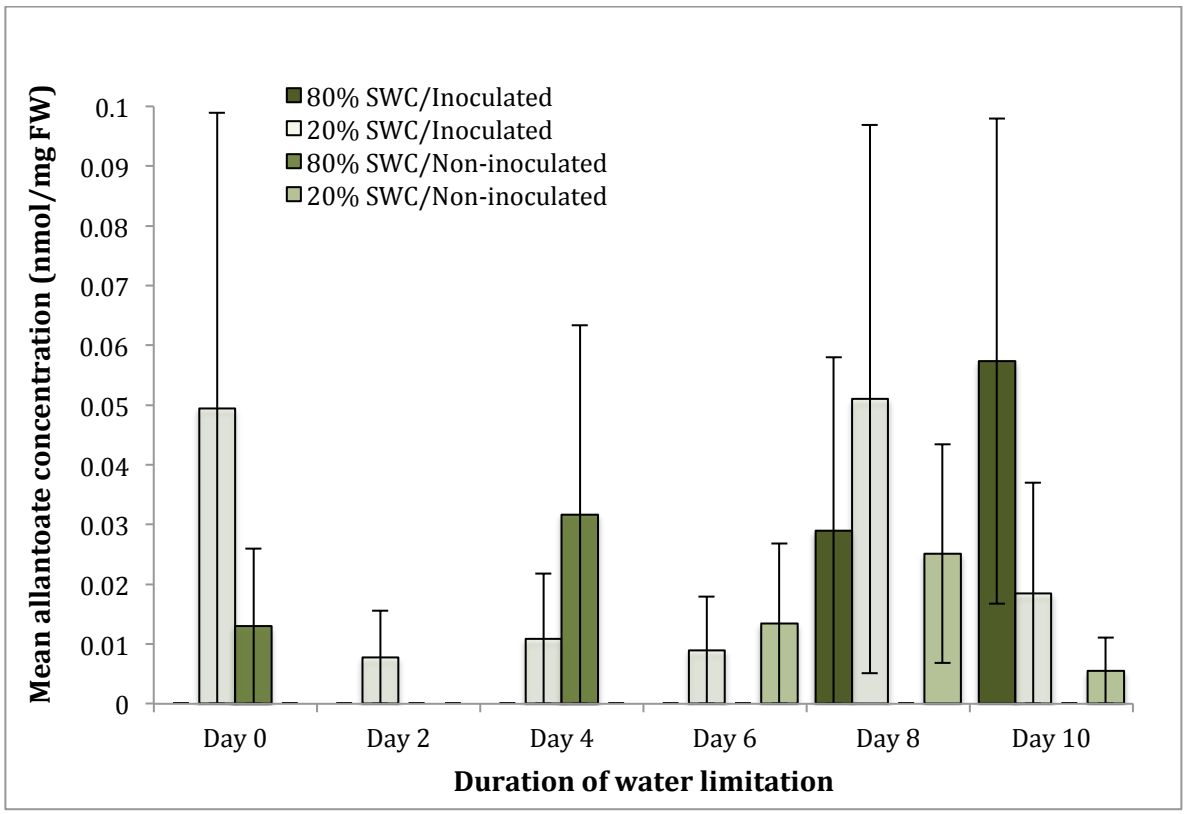

Figure 3.21. Mean allantoate concentration in NPZ4-7540 plants according to treatment types throughout a 10-day water limitation period. Three technical replicates were used per treatment, and the results are represented as the means of three independent experimental repeats (n=3). Error bars represent standard error of the mean.

CHAPTER 4. DISCUSSION

4.1 Accumulation of total ureides in field grown faba bean genotypes

One of the objectives of this thesis was to investigate whether faba bean, a cool season legume and an 'amide exporter', also exports ureides as products of N fixation and whether ureide content differed by genotype. Since ureide accumulation is a sign of high N fixation capability in warm season legumes, high ureide content in a particular genotype may indicate a high N fixing capability in both warm season and cool season legumes (Herridge, 1982).

Charlson et al. (2009) reported that in ureide-exporting legumes like soybean, differences in shoot ureide concentrations among cultivars were evident in drought-sensitive cultivars compared to drought-tolerant genotypes under water limiting conditions (Charlson et al., 2009). Their results showed that at the V7 vegetative growth stage, the drought tolerant genotypes (Jackson) had fewer ureides accumulated, while drought-sensitive (Williams) types had significantly more ureides present in plant tissues. In a response to stress conditions, ureides accumulated in drought-stressed plants. I hypothesized that faba bean may have certain genotypes that are less tolerant to abiotic stress than others, and have increased ureide accumulation which may indicate a genotype with increased drought sensitivity.

The results indicated that there was no significant difference in the concentration of ureides between genotypes used in this study (Table 3.1, 3.3, & 3.5). The fifteen different genotypes represent a narrow genetic base as all of the genotypes come from European and Canadian origin, therefore, all accessions may possess the same physiological mechanisms or adaptations to the cooler Saskatchewan climate. My research was based on plant material from a study aimed at accessing the biomass and nitrogen content in faba bean genotypes as well as the N fixing ability in a dryland prairie environment in order to improve cropping systems through N contribution of faba bean (Dr. Bueckert, unpublished). Since the accessions used in this field study were based on similar parentage,

it would be prudent to design this experiment using different lines of faba bean from a variety of gene pools from different origins, to see if there are any differences in ureide concentration from this wider gene pool. Also, there were only 15 genotypes used in this field experiment, therefore, this study could be expanded to include a much larger base of genotypes to be able to better perceive any differences in ureide content between them. Since only three replications were used in this field experiment, there could also be a Type II error, that there is a difference among genotypes. There could be a failure to detect differences between the genotypes due to low statistical power. It is likely that using a larger genetic pool and more replications could result in differences among genotypes not seen in my research.

Research from Thavarajah et al. (2005) in the cool season legume chickpea, showed that chickpea has an intermediate type of N metabolism in nodules, having both amide and ureide-export (Thavarajah et al., 2005). Chickpea showed moderate levels of both amino acids and ureides throughout the growing season, 213-290 μmol g^{-1} and 1-4 μmol g^{-1} dry leaf tissue, respectively. It was thought that faba bean may also possess this type of intermediate N metabolism and that a high ureide containing genotype would indicate a high N fixing crop and could be used as a screening tool for plant breeding purposes (Herridge, 1982). The concentration of ureides in faba bean leaves (Fig. 3.1, 3.2, & 3.3) was found to be greater than in non-droughted chickpea and pea leaves, which ranged from 1.5 to 8 μmol g^{-1} per leaf (Thavarajah et al., 2005, Thavarajah and Bueckert, unpublished). Faba bean stems had comparable amounts of ureides (Figs. 3.1, 3.2, & 3.3) to non-droughted chickpea stems of around 2 to 5 μmol g^{-1} but less than those in non-droughted pea stems (3 to 15 μmol g^{-1} stem) (Thavarajah et al., 2005, Thavarajah and Bueckert, unpublished). Faba bean leaf concentrations of ureides (10 to 18 μmol g^{-1} DWt) were similar but not as high than those in soybean and stem concentrations of ureides were lower than those in soybean (10 to 30 μmol g^{-1} leaf and 15 to 50 μmol g^{-1} leaf petioles; Purcell et al., 2000). This suggests that faba bean is more similar to classical cool season legumes such as field pea than to warm season legumes like soybean, but faba bean could still possibly be classified as both an amide- and ureide-exporting legume with its similar leaf ureide concentrations to chickpea.

Alamillo et al. (2010) showed that ureides accumulate in common bean tissues in response to drought stress with an increase in *ALN* activity but not *AAH*, indicating that the main cause of allantoate accumulation in leaves is not due to enzyme inhibition of *AAH*. Their results indicated that abscisic acid (ABA) induced ureide gene expression, which infers that a stress response is up-regulating ureide gene expression via ABA. They also found similar results in non-nodulated common bean, indicating that ureide accumulation in water limited common bean is independent from N fixation, and synthesis of ureides *de novo* in root nodules. Recent research in the Dr. Todd laboratory (Biology Dept., University of Saskatchewan, unpublished) indicates that this is a general stress response in all plants. Ureides accumulating in plant tissues in response to abiotic stress conditions have been observed in non-inoculated soybean, common bean, tepary bean, and also in Arabidopsis (Souter, Irani, Todd, unpublished). The presence of ureides in field grown faba bean could also be due to normal purine recycling within plants, or the presence of ureides in faba bean tissue could also be due to a response to an abiotic stress condition, such as water deficit (Serraj et al., 1999; Zrenner et al., 2006) or both.

Ureide concentration varied significantly between plant partitions during the different growth stages. The bar plots (Fig. 3.1, 3.2, 3.3, & 3.4) show that leaf tissue contains the highest concentration of ureides, followed by reproductive parts, nodules, and finally stems. Ureide concentration decreases over the growing season, with anthesis having the highest amount of ureides and physiological maturity having the lowest amount of ureides for leaves, stems, and reproductive parts. However, for nodules, their concentration increased significantly by physiological maturity. Diaz-Leal et al. (2012), found that in both N-fixing and non-fixing common bean, ureides accumulated more towards the end of the growing season, with more ureides present during flowering and mid-pod fill than during initial vegetative growth (Díaz-Leal et al., 2012). These ureides were accumulating due to remobilization of ureides from older plant parts like leaves, to the newer growing areas. They found that ureides did not accumulate in nodules and that gene expression in nodules did not increase over the growth period but that remobilization of nitrogen through *PvALN* was being positively regulated. Nitrogen fixation was found to peak just before the anthesis stage. Their research shows that the level of ureides in both fixing and non-fixing common

bean plants were not affected by the source of N but were regulated by which developmental stage the plant is in, controlled by *PvAAH* gene expression.

Given the low ureide concentration in stems and high amount in leaves in this study, the results also suggest an agreement with studies showing that ureides are not transported from root to shoot in drought stressed *Phaseolus vulgaris* but are synthesized *de novo* in leaves under drought conditions (Alamillo et al., 2010). In another study, ureides were also found to accumulate in nodulated and non-nodulated common bean over the course of the normal growing period (Díaz-Leal et al., 2012). Their results indicate that ureide accumulation in leaves is not related to fixation further along into the growth stage, but is in response to up-regulation of *ALN,* as fixation declines even though ureide accumulation still occurs late in the growing season. This suggests that N is being remobilized from older leaf tissues to newer plant parts, accounting for the increase in ureide accumulation in plant shoots. The bean tissues subjected to water deficit showed increased allantoinase gene expression and enzyme activity, which is most likely regulated developmentally. The results from this research suggest that the presence of ureides in faba bean could possibly be due to synthesis in leaf tissues due to the remobilization of N from older tissues into newer tissues as the plant ages and/or due to a stress response, causing ureides to accumulate as a protective mechanism in plants, but not as a result of ureide production and import from root to shoot.

4.2 Ureide accumulation in water limited faba bean

A water limitation experiment analyzing the amount of individual ureide catabolites, allantoin, uric acid, and allantoate in faba bean leaves was conducted to test the hypothesis that ureides accumulate in both inoculated and non-inoculated water limited plants. This experiment was conducted on two separate genotypes to determine if ureide accumulation differed by genotype. The theory was that water-limited plants would exhibit increased ureide concentration whether they were inoculated or not. Based on the field data, a significantly different response in the two different genotypes was not expected.

When pooled ureide data for each genotype for all treatment types were analyzed by day, the ureides allantoin and allantoate were found to significantly accumulate in the genotype ‘Snowbird’. Allantoin showed a significant increase 8 and 10 days after water

limitation (Fig. 3.10). Allantoate levels were significantly higher between days 6 to 10 (Fig. 3.12). There were no significant differences in uric acid concentration over the 10-day period; however, a general trend of decreasing accumulation was evident (Fig. 3.11). The results for the other genotype, 'NPZ4-7540' were inconclusive and showed no significant differences in ureide accumulation over the water limitation period, though there was a visible trend where allantoin showed a general increase over the water limitation period (Fig. 3.13). No clear trend could be determined from any other measurements.

Ureide content in the water limited faba bean plants were also analyzed according to treatment type; inoculated or non-inoculated conditions as well as a 20% SWC or 80% SWC regime. There were no clear significant effects in inoculated versus uninoculated plants and no significant effects by water treatment. In some treatments ureides were higher in inoculated plants and in other cases, ureides were higher in uninoculated plants, and the same for the water treatments. Therefore, the results from these analyses were inconclusive. There were a few significant effects in some treatments for both genotypes, however, the effects were not evident over the entire course of the water limitation period and were somewhat random (Fig. 3.16, 3.17, 3.18, & 3.19). There were significantly more ureides in the inoculated Snowbird leaves compared to non-inoculated plants 8 days after water limitation (Fig. 3.16). Uric acid content was also significantly different by water treatment at day 8 of water limitation (Fig. 3.17). No significant trends were observed in the quantification of allantoate, however, a general trend can be observed in the final four days of the experiment, where, the greatest allantoate accumulation occurred in well-watered inoculated plants, followed by the low-watered non-inoculated plants (Fig. 3.18).

In the genotype NPZ4-7540, a significant effect of inoculation was observed at days 8 and 10 of water limitation for allantoin concentration (Fig. 3.19). Here the amount of allantoin was significantly greater in inoculated versus non-inoculated plants. These results may indicate that inoculated plants that were less healthy than their non-inoculated counterparts had greater accumulation of ureides since these plants were more stressed than the non-inoculated plants. There was also a significant interaction observed between inoculation and water treatment for uric acid at day 2 of the experiment, where significantly more uric acid was present in the non-inoculated tissues than inoculated tissues, and when factoring in water treatment, significantly more ureides were present in

well-watered versus low-watered tissues (Fig. 3.20). A possible explanation for these results could be that the low-watered plants were the unhealthiest of the four treatments and were severely stressed and therefore, little ureides could be detected in the tissues analyzed. There were no significant differences observed in allantoate accumulation in NPZ4-7540 (Fig. 3.21).

The inconclusive results in the water limitation experiment were most likely due to the overall health of the plants. As seen in Fig. 3.8 and 3.9, the inoculated plants were chlorotic and exhibited symptoms of N deficiency compared to their uninoculated counterparts based on visual observation. The inoculated plants were also visually smaller in size, with a shorter shoot biomass than the uninoculated plants. Root nodules were also assessed and root nodules in inoculated plants were found to be small in number and mostly pale in color. Most likely, the bacteria in the nodules were not fixing nitrogen well or at all. This could have been due to plants being overwatered and the Sunshine Gro Mix holding too much water and being oversaturated. The uninoculatd plants were visibly greener, larger, and healthier and were most likely not N starved like the inoculated plants. Since plants health was variable by treatment this could have led to inconclusive results in regards to ureide accumulation. In other words, ureide accumulation is most likely based on the overall condition of the plant, which would include the apparent nitrogen deficiency and possible waterlogged condition in the inoculated plants. Also, a noticeable change was observed in plant health at around three and a half weeks after being transplanted. All plants had roughly the same health status until three weeks old, following this, noticeable changes were evident and the inoculated plants began to decline in overall health compared to uninoculated plants.

4.2.1 Recommendations for water limitation experiment

In order to investigate the accumulation of ureides in faba bean further, another water limitation experiment could be carried out where some variables could be adjusted to account for errors that occurred in the first experiment. The 80% SWC content used in the experiment may have been too high for faba bean. Faba bean prefers drier soil conditions than 80% SWC in growth chamber experiments (Dr. Bueckert, personal communication) and keeping plants at 80% SWC may have caused them to become

waterlogged. In the inoculated plants, this waterlogged condition may have caused nodules to be in an anoxic state inhibiting N fixation. This is a likely cause of the plants' pale and yellow condition, most probably indicative of N deficiency. While observing the nodules of inoculated plant roots, nodules appeared few in number and in some specimens, many of the nodules were pale in color. It is also possible that the inoculant used was not specific enough for faba bean or that the inoculant was inactive and nodulation was ineffective. Soil conditions could be a possible explanation for this also. Lack of effective nodulation or poor nodule conditions could have caused the faba bean plants to not actually be fixing N, leading to N deficient and stressed plants. Since there is no specific inoculant for faba bean, a more specific inoculant should be developed by the industry that is highly specific to faba bean to allow for better inoculation and therefore, better N fixation, leading to healthier plants.

Also, this water limitation experiment should be repeated starting on three week-old faba bean plants, as their overall health was better in younger plants based on my observations. Plants began to show signs of stress from N deficiency at around 3.5 weeks of age. Differences in inoculated versus uninoculated plants became even more evident from this age onwards. Prior to this, all plants had roughly the same health status based on visual observation (Fig. 3.16 & 3.17). Starting the water limitation experiment at around 3 to 3.5 weeks of age would have allowed for better observation of the water limitation response in healthier plants. It is possible that the response of ureide accumulation in stressed plants could have been more apparent if plants were in a better state of health and not N starved or senescing.

4.3 Gene identification and quantification of ureide catabolic genes

Gene transcripts for allantoinase (*VfALN*) and allantoate amidohydrolase (*VfAAH*) were identified in faba bean based on known sequences from soybean and barrel clover. These two genes were chosen because they metabolize the major purine metabolites allantoin and allantoate, which accumulate in stressed plant tissues and are thought to be involved in protecting plants from abiotic stress (Alamillo et al., 2010; Werner and Witte, 2011; Watanabe et al., 2013). However, expression of these genes was not assessed in the plant tissues from the water limitation experiment using RT-PCR as previously planned,

since the results of this experiment were inconclusive as to the accumulation of ureides over a 10-day water limitation period.

My initial expectation was that I would see a clear difference in ureide concentration in the low-watered plant tissues and I expected to see no difference between the inoculated and non-inoculated plants, since faba bean has been classified as an amide-exporting legume. I expected to see differences in well-watered and low-watered plants only. The results from a study by Charlson et al. (2009) coincide with other results that indicate that expression of *AAH* in soybean is independent of tolerance to drought (Charlson et al., 2009). These authors demonstrated that the drought-tolerant varieties had less ureides than the drought-sensitive ones, while gene expression for *AAH* was similar in all treatments, suggesting that gene expression is not responsible for the increased ureide levels present in soybean. Their research also indicated the ureides accumulate due to a stress response and not due to N fixation in water limited plants. Since I did not see these differences clearly in my experiment, I decided against pursuing the gene expression analysis, as there was no clear treatment or time point that demonstrated an obvious response in my water limitation experiment. Any differences in gene expression observed would have been difficult to interpret in light of the ureide data. However, the sequences and primers generated are now available to address allantoinase and allantoate amidohydrolase gene expression changes in future experiments in faba bean.

CHAPTER 5. REFERENCES

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APPENDIX A

INITIAL ANALYSIS OF UREIDE CONCENTRATION IN FIELD GROWN FABA BEAN TO OBSERVE NORMALITY OF THE DATA.

Using a histogram of *ureides* at growth stage *mid-pod,* the data showed a histogram skewed to the left, which meant the data did not follow a normal distribution (Fig. A.1A). Since the data were not normally distributed, the data were log (natural logarithm) transformed and a histogram of the transformed data showed a bell-shaped curve with a normal distribution (Fig. A.1B). The qqnorm function was used to determine the normality of the data, comparing the ranked samples from growth stage *mid-pod* from the distribution against a similar number of theoretical ranked quantiles taken from a normal distribution (Fig. A.2A). Since the data were not normally distributed, the plot showed a non-linear, S-shaped curve instead of a straight line. Once log transformed, the distribution of the data improved, showing a more linear curve (Fig. A.2B).

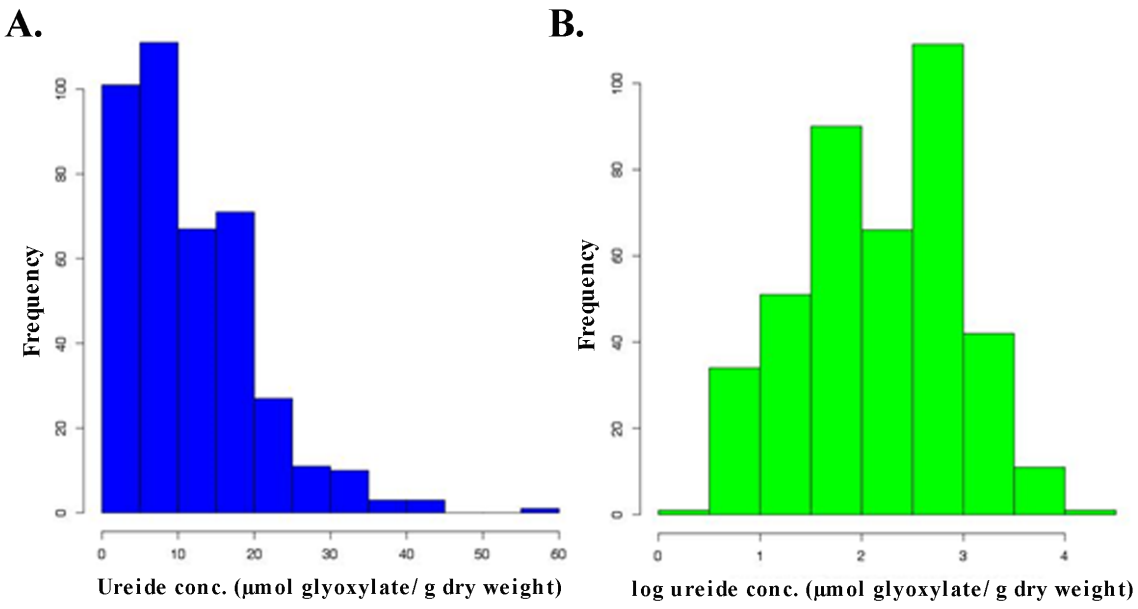

Figure A.1. Checking the normal distribution of the data at growth stage *mid-pod*. **A.)** Histogram of average ureide concentration at growth stage *mid-pod* (left), and **B.)** log transformed data with a normalized distribution (right).

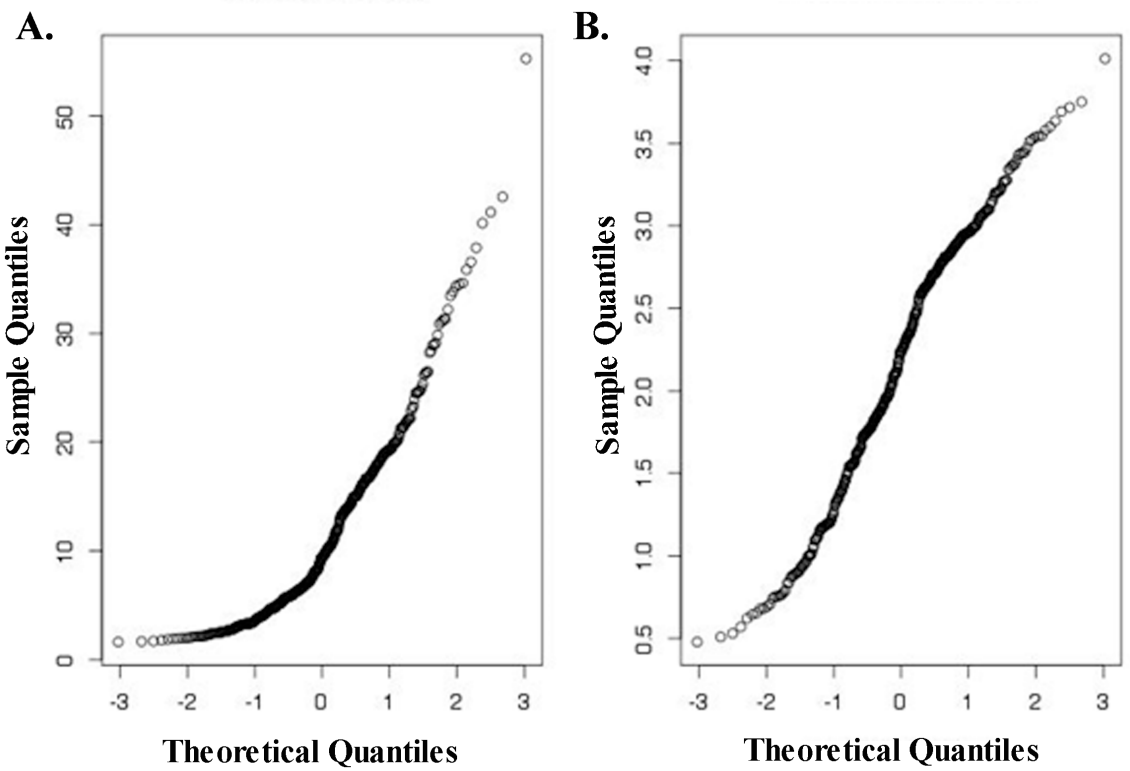

Figure A.2. Checking the normal distribution of the data at growth stage *mid-pod*. **A.)** qqnorm plot of average ureide concentration using data from the *mid-pod* growth stage, showing an S-shaped curve (left), **B.)** qqnorm plot of log-transformed data showing a more linear curve indicating a nearly normal distribution (right).

APPENDIX B

STATISTICAL ANALYSIS OF UREIDE CONCENTRATION FOR FIELD GROWN FABA BEAN AT GROWTH STAGE ANTHESIS

The following tables show the initial data analysis and model checking tools used to find the best minimally adequate model to explain the data.

Table B.1. Variance components analysis for the mixed model at stage *Anthesis*, showing most of the variation in the data is due to *plant partition.*

Factor	Standard Deviation	Standard Error
Location.Year	0.224	8.82
Block	7.705×10^{-5}	1.03×10^{-6}
Genotype	8.044×10^{-6}	1.13×10^{-8}
Plant Partition	0.721	91.20
Residuals	0.004	0.002

Table B.2. AIC values for the three mixed models tested, resulting in the selection of model '3' with the lowest AIC value.

Model	DF	AIC
1	49	215.5
2	21	103.1
3	7	34.1

Next, to investigate how well the model describes the data, the standardized residuals versus fitted values were graphed using model Model 3 to check if model selection parameters were suitable. Fig. B.1 shows a reasonably linear function of the fitted values for the response variable *loganthesis* (log transformed *ureides*) against a standard model indicating good model fitness. The residuals vs. fitted values show homogeneity of variance with a relatively even distribution of the residuals with no obvious fan-shape pattern, therefore, we can conclude that the model is fitting the values adequately (Fig. B.2). The qqnorm plot of the residuals shows that the residuals are following a normal distribution in a near linear curve (Fig. B.3). The model is showing less goodness of fit at

the high and low values due to the residuals being a bit unevenly distributed, not completely linear, but overall, the errors are close to normally distributed. Qqnorm plots for the random factor *location x year,* also followed similar linear curves with less goodness of fit at the high and low fitted values, but overall indicating the model is fitting the data adequately (Fig. B.4 & B.5)

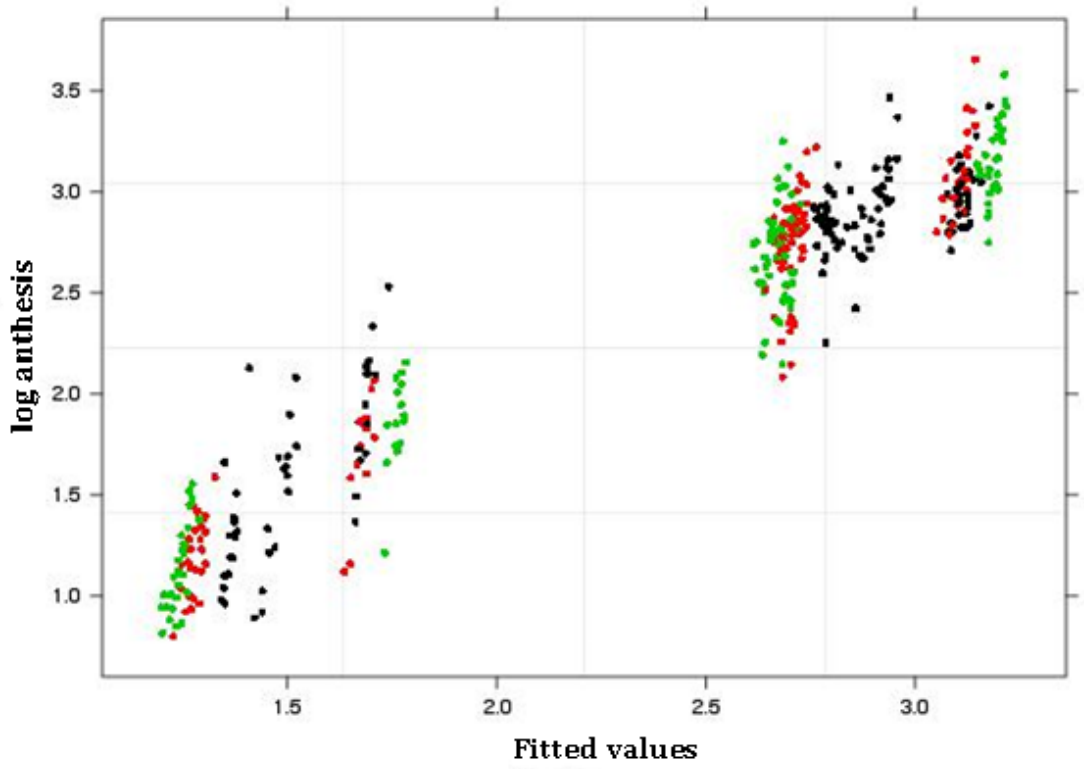

Figure B.1. Model checking graph showing the response variable *loganthesis* showing a reasonably linear function of the fitted values to a standard model.

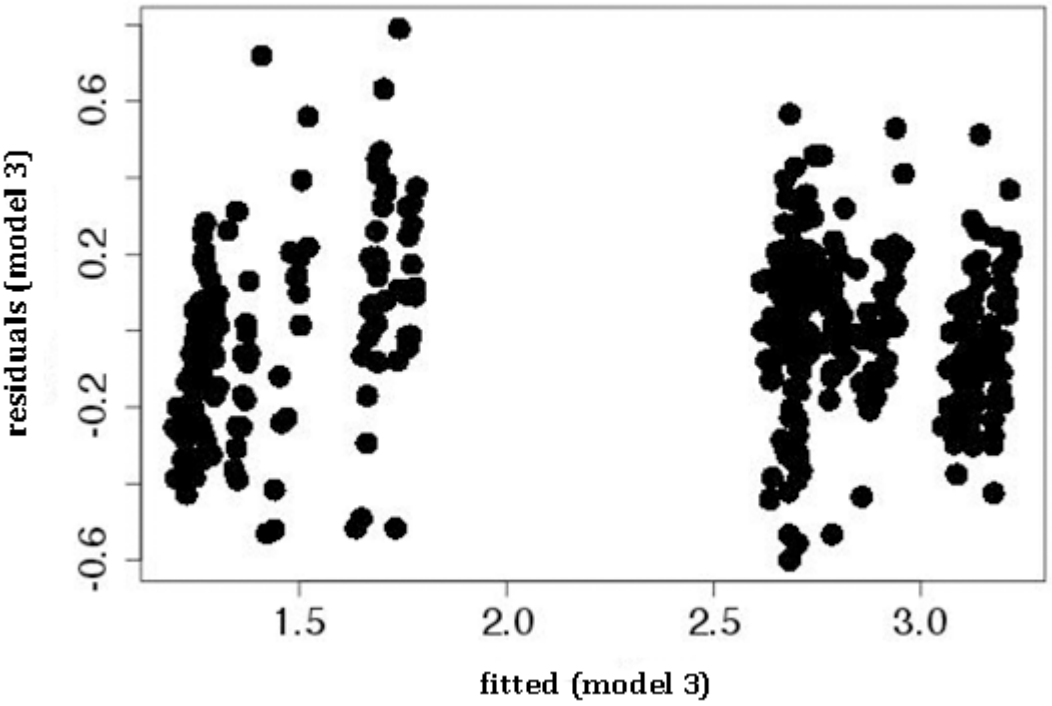

Figure B.2. The standardized residuals against fitted values of Model 3, show a relatively even distribution.

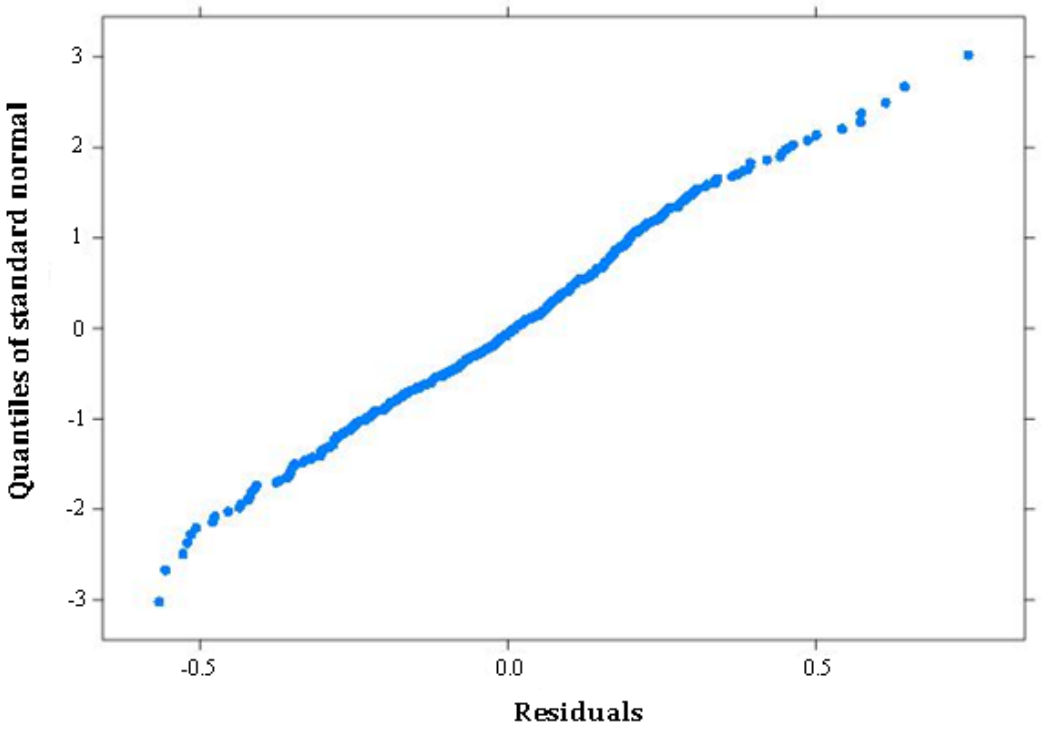

Figure B.4. The residuals follow a relatively normal distribution in this qqnorm plot at *Anthesis* with a nearly straight line.

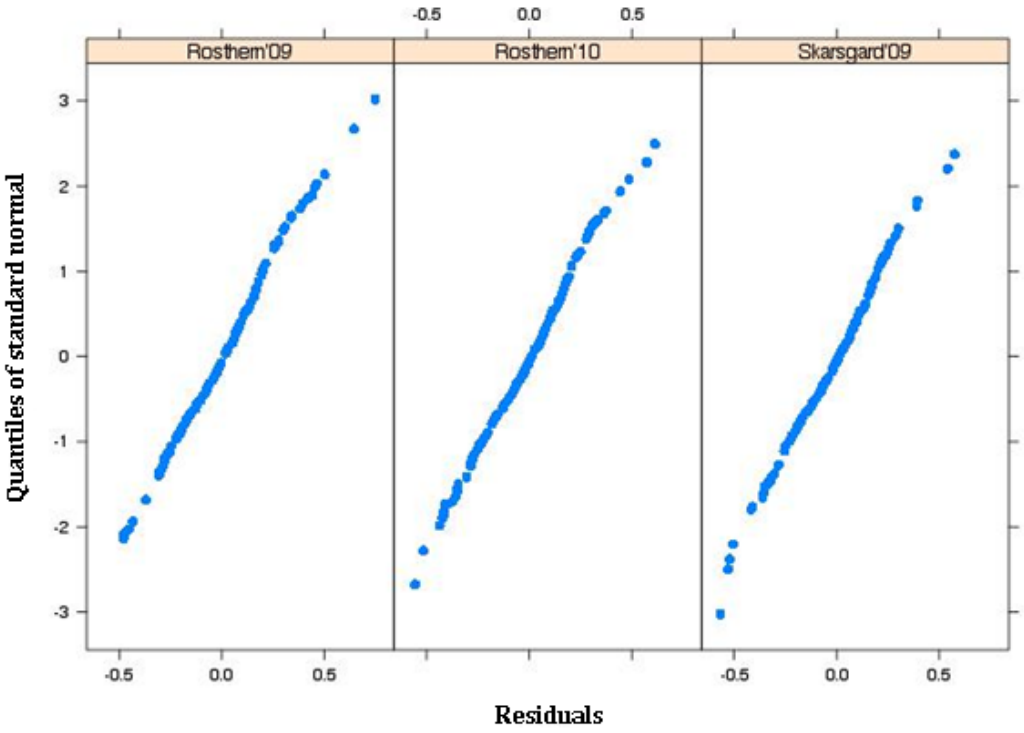

Figure B.5. The errors (residuals) from the random factor *location x year* also follow an adequate normal distribution with a straight line.

APPENDIX C

ANALYSIS OF UREIDE CONCENTRATION AT GROWTH STAGE, MID-POD.

The variance in the data is contributed 94.8% by *plant partition,* 5.3% by *location x year,* 0.0014% by residuals, <1% by *block* and <<<1% by *genotype* (Table C.1). AIC values show that again the model with the *genotype x plant partition* interaction removed (Model 2, AIC= 573.6) is a better model than with this interaction included (Model 1, AIC= 653.7), and finally with *genotype* removed from the fixed effects in the model (Model 3) we see the lowest AIC value (513.9), therefore, this is the minimally adequate model (Table C.2).

Table C.1. Variance components analysis for the mixed model at growth stage *mid-pod* indicating the greatest amount of variance in the data is due to *plant partition*.

Factors	Standard Deviation	Standard Error
Location.Year	0.178	5.181
Block	1.198×10^{-5}	2.342×10^{-8}
Genotype	1.696×10^{-5}	4.690×10^{-8}
Plant Partition	0.762	94.800
Residuals	0.004	0.001

Table C.2. AIC values for the three mixed models tested, resulting in the selection of 'Model 3'.

Model	DF	AIC
1	49	653.7
2	21	573.6
3	7	513.9

APPENDIX D

ANALYSIS OF UREIDE CONCENTRATION AT GROWTH STAGE PHYSIOLOGICAL MATURITY

Initial data analysis looking at the variance in the data indicated most of the variance of the data is due to *plant partition* at 97.3%, 2.4% due to *location x year,* 0.0926% due to residuals, <1% from *block*, and <<<1% due to *genotype* (Table D.1). Based on the AIC values for the models run, Model 3 was chosen as the minimally adequate model since all non-significant terms were removed and it had the lowest AIC value (Table D.2).

Table D.1. Variance components analysis for the mixed model at *physiological maturity*, showing the greatest amount of variance in the data due to *plant partition*.

Factors	Standard Deviation	Standard Error
Location.Year	0.107	2.401
Block	0.251	2.342×10^{-8}
Genotype	8.051×10^{-8}	4.690×10^{-8}
Plant.Partition	0.682	97.300
Residuals	0.021	0.093

Table D.2. AIC values for the three mixed models tested, resulting in the selection of 'Model 3'.

Model	DF	AIC
1	64	776.1
2	22	665.1
3	8	602.6

APPENDIX E

ANALYSIS OF UREIDES IN FIELD GROWN FABA BEAN NODULE TISSUE

Based on the 'lme' model accounting for all random variables, the variance components analysis indicated the greatest amount of variance in the data was due to *time* at 88.8%, 5.6% *location*, 5.6% *year*, <1% *block*, <1% *genotype*, and <1% residuals (Table E.1). Finally, to check that the model describes the data adequately, model checking was used as previously described. All model checking graphs showed that the model chosen was adequately describing the data.

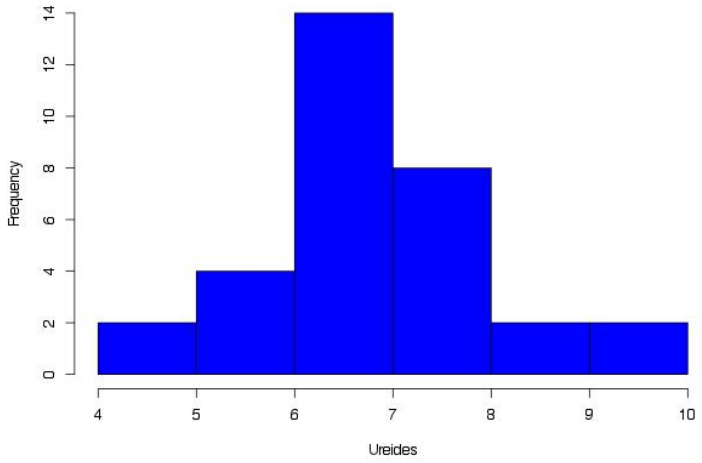

Figure E.1. Histogram showing the normal distribution of ureides in nodule samples.

Table E.1. Variance components analysis for the mixed model 'lme' of ureides found in the nodule field samples indicating the greatest amount of variance in the data is due to the factor *time*.

Factor	Standard Deviation	Standard Error
Location	0.272	5.615
Year	0.272	5.615
Block	4.481×10^{-5}	1.522×10^{-7}
Genotype	1.456×10^{-5}	1.607×10^{-8}
Time	1.082	88.763
Residuals	0.009	6.539×10^{-3}

Table E.2. AIC values using the different models and the degrees of freedom for each model. Model #4 has the lowest AIC and is therefore the best-fit model for the data.

Model	df	AIC
1	10	100.1
2	9	101.1
3	8	99.1
4	7	97.1

APPENDIX F

ANALYSIS OF UREIDE CONCENTRATION IN CHAMBER GROWN FABA BEAN UNDER WATER LIMITING CONDITIONS

Faba bean plants were subjected to a water limitation experiment for 10 days after reaching 35 days old. Plants from two genotypes (Snowbird and NPZ4-7540) and each treatment (either inoculated and well-watered or low-watered or non-inoculated and well-watered or low-watered) were analyzed for individual ureide catabolites. The data was compared between cultivars to see if there were any significant differences in ureides by day for each compound. The majority of the data showed no significant difference in ureides by day between the two genotypes therefore, the ureides were analyzed separately by cultivar in further analyses.

Table F.1. Data from the Mann Whitney U test, comparing the difference in ureides for both cultivars by day. The table contains p-values for each comparison. Values that are significantly different ($p < 0.05$) are in bold.

Water Limitation	Allantoin	Uric Acid	Allantoate
Day 0	0.317	0.727	0.080
Day 2	0.154	**0.005**	0.132
Day 4	**0.033**	0.501	0.132
Day 6	**0.021**	0.331	**0.0001**
Day 8	0.414	0.081	**0.0001**
Day 10	0.191	**0.035**	**0.0001**