UREIDE ACCUMULATION DURING ABIOTIC STRESS IN LEGUMES

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Abstract

Allantoin and allantoate, ureides formed during the catabolism of purines, accumulate in plant tissue in response to drought and other abiotic stresses. Allantoin, allantoate, and a precursor molecule, uric acid, have been hypothesized to be scavengers of the reactive oxygen species (ROS) formed during abiotic stress. This research investigated whether a direct link exists between these ureides and the ROS produced during water limitation, sub-zero temperature stress, and induced oxidative stress. Allantoin and allantoate accumulated in both nitrogen-fixing and nitrogen-fertilized soybean leaves during water limitation. The accumulation of ureides in the leaves could also be generated by treatment with methyl viologen (MV) which induces superoxide production. Isolated leaf disk experiments demonstrated that allantoin did not directly scavenge ROS, but stimulated a response in the plant that indirectly decreased ROS. In contrast, uric acid had a direct chemical interaction with hydrogen peroxide (H_2O_2). Both allantoin and uric acid decreased cell death in leaf tissue after treatment with H_2O_2 and allantoin decreased cell death after treatment with MV. In addition, I examined whether the accumulation of ureides could be used to predict abiotic stress tolerance of different genotypes in a population of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean. Sub-zero temperature stress and water limitation stress tolerance were correlated with ureide content in a population of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean, however these correlations were too weak for implementation of ureide accumulation quantification as a plant breeding tool. Results of this thesis will aid in understanding ureide accumulation during abiotic stress, will assist in determining the function of ureide accumulation during abiotic stress, and will serve as the starting point to bridge the gap between the mechanistic knowledge of ureide accumulation during abiotic stress and applying it in larger scale agricultural scenarios.

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Dedication

The only reason I have achieved all that I have is because of my parents and the sacrifices they have made in their lives to give me and my sisters the opportunities they never had. For that and for all your love, thank you Mom and Dad.

This is for you.

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List of Abbreviations

$^{15}N_2$	isotopically labelled dinitrogen
AAH	allantoate amidohydrolase
ABA	abscisic acid
ALN	allantoinase
AMP	adenosine monophosphate
ANOVA	analysis of variance
$BC_{(x)}$	back cross generation x
BCA	bicinchoninic acid
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary DNA
CDS	coding sequence
DAB	3,3'diaminobenzidine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DW	dry weight
EC	electrical conductivity
EL	electrolyte leakage
ER	endoplasmic reticulum
$F_{(x)}$	filial generation x
FAO	Food and Agriculture Organization
PC	pot capacity
FW	fresh weight
GMP	guanosine monophosphate
GS/GOGAT	glutamine synthestase/glutamine oxoglutarate aminotransferase
H_2O_2	hydrogen peroxide

HIU	5-hydroxyisourate
HPLC	High Performance Liquid Chromatography
IMP	inosine monophosphate
LD	leaf disk
LD_{50}	lethal dose, 50%
MDA	malonic dialdehyde
MV	methyl viologen
NAD^+	nicotinamide adenine dinucleotide
NBT	nitroblue tetrazolium
NH_4	ammonium
O_2^-	superoxide
OHCU	2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline
PCR	polymerase chain reaction
PSII	photosystem II
PTFE	polytetrafluoroethylene
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
RuBP	ribulose-1,5- bisphosphate
SARK	senescence associated receptor-like kinase
TMOP	1,1,3,3-tetramethoxypropane
Tris	tris(hydroxymethyl)aminomethane
UP	ureide permease
USDA-ARS	United States Department of Agriculture – Agriculture Research Service
XDH	xanthine dehydrogenase
XMP	xanthosine monophosphate

1. Introduction and Literature Review

1.1. General introduction

Nitrogen is an essential nutrient for plant growth, development and reproduction; however, plants are often limited by nitrogen availability. Plants do not contain nitrogenase, an enzyme that breaks the triple bond of atmospheric nitrogen (N_2). Instead, plants have to take up nitrogen from the environment primarily in the form of ammonium (NH_4^+), nitrate (NO_3), or amino acids (Nasholm et al., 2009). Additionally, leguminous and actinorhizal plants have developed a symbiotic relationship with specific bacteria able to break atmospheric nitrogen, thereby contributing another nitrogen source to these plants (Schubert, 1986; Franche et al., 2009). In this symbiotic relationship, the N_2 is reduced to ammonia by the bacteria and is released to the plant in exchange for carbon from photosynthate. To be transported through the plant, the ammonia must be converted to an organic form. Pea (*Pisum sativum*), alfalfa (*Medicago sativa*) and faba bean (*Vicia faba*), transport nitrogen primarily in the form of amide amino acids. Members of the *Glycine*, *Phaseolus* and *Vigna* species transport nitrogen as ureides (Herridge et al., 1978; Schubert, 1986). In the 1980s, the difference in nitrogen export was associated with the predicted origin of these legumes: the amide exporting legumes from the temperate regions and the ureide exporting legumes from the tropical/subtropical regions (Sprent, 1980). As such, when discussing ureide metabolism, *Glycine*, *Phaseolus* and *Vigna* have since been referred to as tropical legumes (and will be in the remainder of this thesis).

Ureides, molecules containing a urea group, are not only products of nitrogen fixation, but are primarily produced in plants as the breakdown product of purines (Schubert, 1981). By catabolizing purines into ureides, and ultimately back to NH_4^+, the plant can recover the nitrogen and remobilize it for use in other parts of the plant (Werner et al., 2013). Remobilization can occur in senescing tissues because of age, lifecycle stage, or in response to abiotic and biotic stress (Masclaux-Daubresse et al., 2010).

An abiotic stress is any environmental factor that reduces the growth, survival, or fecundity of a plant (Maestre et al., 2005). Of particular interest in this thesis is the observation that ureides

accumulate in soybean and bean plants during multiple abiotic stresses. Ureide accumulation has also been shown to occur in non-fixing legumes and non-legumes (Brychkova et al., 2008; Alamillo et al., 2010).

Since ureides accumulate during multiple stresses, they are likely responding to a common factor. It has been hypothesized that ureides are accumulating to scavenge reactive oxygen species (ROS) produced during abiotic stress (Brychkova et al., 2008). Uric acid, a ureide, acts as an antioxidant in humans (Ames et al., 1981) and it is possible it plays a similar role in plants. In addition, it has been hypothesised that the breakdown product of uric acid, allantoin, is also an antioxidant (Gus'kov et al., 2004), however this hypothesis has been questioned, at least in a direct antioxidant role (Wang et al., 2012). Nonetheless, allantoin has been shown to play an alternate role in stress response, possibly by activating abscisic acid (ABA) production (Watanabe et al., 2013).

Abiotic stresses severely limit the yield potential of crops worldwide (Boyer, 1982). The Food and Agriculture Organization (FAO) estimates that only 3.5% of the land surface of the earth does not have a climatic constraint to crop production (van Velthuizen et al., 2007). These constraints will only worsen with the changes being predicted due to climate change (Morton, 2007). Therefore, it is essential to breed for crops that are better able to withstand abiotic stresses.

Common bean is one such crop species that requires increased abiotic stress tolerance. Common bean is the most important grain legume for human consumption (Schmutz et al., 2014) and plays an import role in diets worldwide, especially in developing countries (Messina, 1999). However, currently available common bean varieties lack abiotic stress tolerance. Presently, abiotic and biotic stresses are reducing bean yields in developing countries by 1/3 of what yields are in more favourable environments (Porch et al., 2013). It is estimated that to keep up with population growth, a 30% yield increase over current yields is needed by 2050 (Porch et al., 2013).

As the genetic diversity in the currently marketed bean classes is narrow, introducing abiotic stress resistance through wild relatives may be a key to improving abiotic stress resistance in bean (Rao et al., 2013). Tepary bean, *Phaseolus acutifolius*, has been shown to be resistant to many abiotic stresses, including high temperature stress (Gaur et al., 2015), sub-zero temperature stress (Balasubramanian et al., 2004a), salt stress (Goertz and Coons, 1991), and drought stress (Beebe et al., 2013b). As a relative of common bean (Delgado-Salinas et al., 2006), tepary bean may be able to be utilized as a donor of abiotic stress tolerance genes for common bean.

Ureides accumulate to different extents in different cultivars of soybean (Serraj and Sinclair, 1996; Purcell et al., 1998). As such, it is possible that ureide accumulation could serve as a proxy indicator of abiotic stress and become a simple tool to determine the stress resistance differences between genotypes. Gaps still remain in the understanding of ureide metabolism in plants during conditions of abiotic stress. Gaining insight into the changes in ureide metabolism and ureide accumulation during abiotic stress could potentially assist in selecting for or developing plant genotypes better able to tolerate abiotic stress.

1.2. Literature review

1.2.1. Ureides

1.2.1.1. Ureides from purine metabolism

Ureides, molecules containing a urea group, are found in all organisms, including animals, plants, fungi, algae, and bacteria, as products of purine catabolism and purine nitrogen scavenging (Schubert, 1981; Todd et al., 2006; Zrenner et al., 2006). Plants differ from animals in their purine catabolism, in that plants are able to completely catabolize purines to recycle the nitrogen whereas animals excrete partially degraded purines (Vogels and Van Der Drift, 1976). Plants are sessile organisms with scarce nitrogen availability and the ability to recycle all available nitrogen is important for proper growth and reproduction (Werner and Witte, 2011), whereas in animals excretion of excess nitrogen is an important physiological process.

Purines, heterocyclic aromatic compounds primarily known as components of DNA, RNA, ATP, NADH and coenzyme A, can be degraded to release the nitrogen atoms bound in these molecules (Boldt and Zrenner, 2003; Moffatt and Ashihara, 2002). During purine scavenging in plants, both adenosine monophosphate (AMP) and guanosine monophosphate (GMP) are degraded to form xanthine, but through two different pathways (Figure 1.1). AMP is deaminated to inosine monophosphate (IMP) by AMP deaminase (EC: 3.5.4.6). IMP is dephosphorylated to yield inosine which is then hydrolyzed to form hypoxanthine by IMP dehydrogenase (EC: 1.1.1.205) and 5’-nucleotidase (EC: 3.1.3.5), respectively (Atkins, 1981; Shelp and Atkins, 1983).

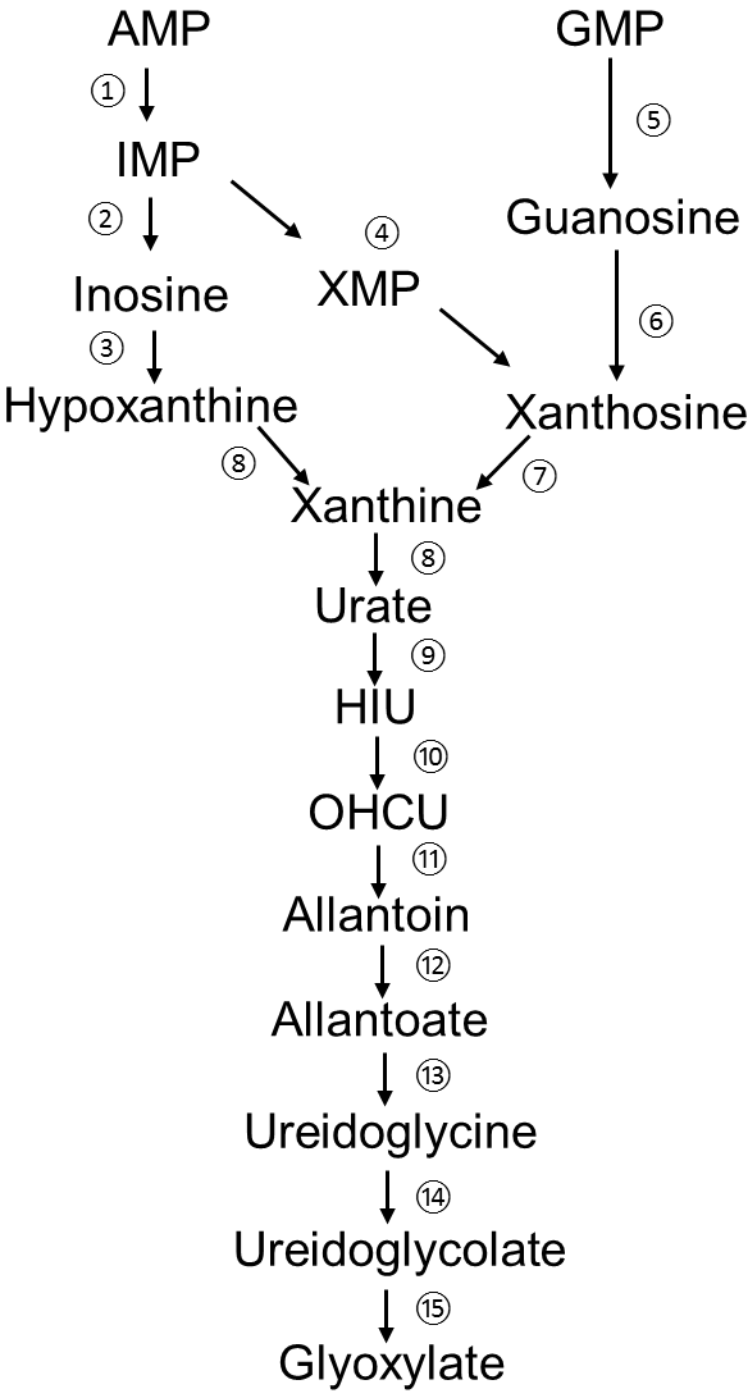


Figure 1.1: The catabolism of the purines adenosine monophosphate and guanosine monophosphate in plants. Enzymes catalyzing reactions include (1) adenosine monophosphate (AMP) deaminase (2) inosine monophosphate (IMP) dehydrogenase (3) 5'-nucleotidase (4) IMP dehydrogenase (5) guanosine monophosphate (GMP) deaminase (6) guanine deaminase (7) purine nucleosidase (8) xanthine dehydrogenase (9) uricase (10) 5-hydroxyisourate (HIU) hydrolase (11) 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) decarboxylase (12) allantoinase (13) allantoate amidohydrolase (14) ureidoglycine aminohydrolase (15) ureidoglycolate amidohydrolase.

Hypoxathine is further broken down to form xanthine by xanthine dehydrogenase (EC: 1.17.3.2). In contrast to AMP, GMP is converted to xanthine through the intermediates of guanosine and xanthosine by guanosine deaminase (EC: 3.5.4.15), guanine deaminase (EC: 3.5.4.3) and purine nucleosidase (EC: 3.2.2.1) (Dahncke and Witte, 2013; Moffatt and Ashihara, 2002). A proposed second route of IMP degradation may also occur in soybean nodules, in which IMP is converted to xanthosine monophosphate (XMP), then dephosphorylated to form xanthosine (Moffatt and Ashihara, 2002).

After the formation of xanthine, the subsequent steps of purine catabolism are the same irrespective of the initial purine molecule (Figure 1.1). Xanthine is converted to urate by xanthine dehydrogenase (EC: 1.17.1.4) (Triplett et al., 1982). Urate is converted into allantoin via 5-hydroxyisourate (HIU) and 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU) intermediates, through the action of uricase (EC: 1.7.3.3), HIU hydrolase (EC: 3.5.2.17) and OHCU decarboxylase (EC: 4.1.1.97) (Ramazzina et al., 2006; Tipton, 2006). However, in Arabidopsis it has been shown that the hydrolysis of HIU and the decarboxylation of OHCU is controlled by a bifunctional enzyme that catalyzes both reactions, called allantoin synthase (Lamberto et al., 2010). Allantoin is catabolized by the enzyme allantoinase (EC: 3.5.2.5) into allantoate (Figure 1.1 and 1.2) (Webb and Lindell, 1993). The first nitrogen atom is released as ammonia when allantoate is broken down into ureidoglycine by allantoate amidohydrolase (EC: 3.5.3.9) (Todd and Polacco, 2006). A second ammonia molecule is released in the conversion of ureidoglycine to ureidoglycolate by ureidoglycine aminohydrolase (EC: 3.5.3.26). The final step of ureide breakdown in plants is the catabolism of ureidoglycolate, releasing two molecules of ammonia and one molecule of glyoxylate, catalyzed by ureidoglycolate amidohydrolase (EC: 3.5.1.116) (Werner et al., 2010).

1.2.1.2. Ureides from nitrogen fixation

Tropical legumes, like soybean, bean and cowpea, have an additional source of ureides in addition to those ureides produced during purine remobilization. By supplying $^{15}N_2$ to soybean plants, Matsumoto et al. (1977) showed that allantoin was formed in the nodules of soybean plants during nitrogen fixation and was eventually transported to developing seeds. McClure and Israel

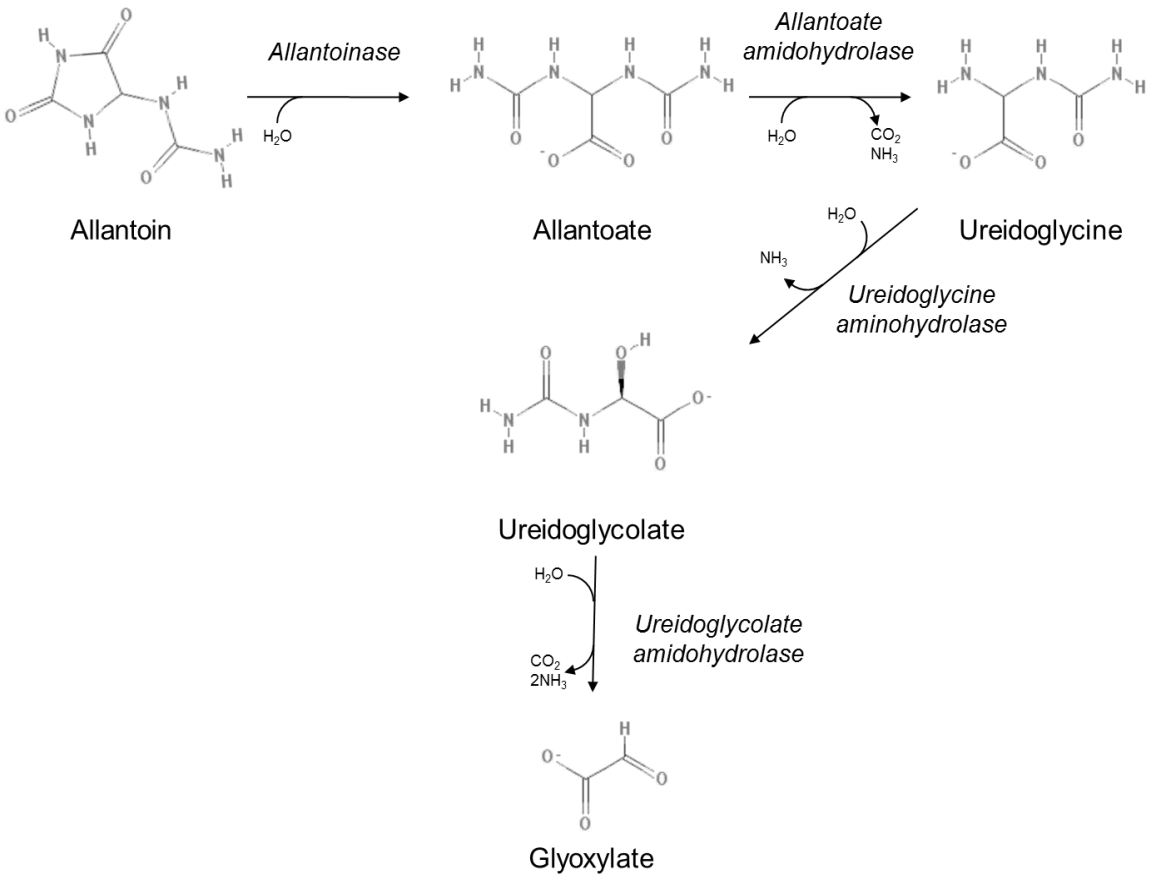


Figure 1.2: The ureide degradation pathway. Allantoin is broken down into glyoxylate yielding four molecules of ammonia.

(1979) characterized the distribution of nitrogen in the xylem sap of nitrogen-fixing soybean and determined 78% of the total nitrogen was in the form of ureides in nitrogen-fixing soybean compared to 6% of total nitrogen in the form of ureides in nitrogen-fertilized soybean. The dominant ureide was allantoic acid (McClure and Israel, 1979). Through these studies, the researchers determined that soybean converted fixed nitrogen into ureides and that ureides were the main form of nitrogen transported in soybean.

Ammonia, the product of nitrogen fixation, must be converted to an organic form to be transported throughout the plant because it becomes toxic at higher concentrations (Schubert, 1986). In tropical legumes, allantoin and allantoate function as the primary transport molecules for fixed nitrogen (Schubert, 1981; Thomas and Schrader, 1981). Ammonia is first incorporated into glutamine and glutamate by the GS/GOGAT (glutamine synthestase/glutamine oxoglutarate aminotransferase) cycle. Glutamate and its transamination product, aspartate, are incorporated into purines, which are oxidatively degraded to form allantoin and allantoate (Schuller et al., 1986). Enzyme activity suggests all reactions up to xanthine occur in the infected cells of the nodule (Reynolds et al., 1982; Schubert, 1986), after which the breakdown of uric acid and downstream metabolites occurs in the uninfected cells of the nodule (Kouchi et al., 1988). Xanthine dehydrogenase is detected in both infected and uninfected cells (Tajima et al., 2004).

From the nodules, allantoin and allantoate are transported to the sink tissues of the plant via the xylem. In soybean, allantoin and allantoate are transported from the nodule by ureide permease, located in the plasma membrane of the cell (Collier and Tegeder, 2012). Ureide permease has also been found in Arabidopsis (Schmidt et al., 2004) and common bean (Pelissier et al., 2004), however in these plants the ureide permease was only shown to transport allantoin.

For the sink tissues of the plant to benefit from the nitrogen fixed during nitrogen fixation the ureides must be broken down to ammonia and re-assimilated. The breakdown of ureides from nitrogen fixation follows the same catabolic pathway as the breakdown of ureides synthesized as a result of purine remobilization and releases glyoxylate, two molecules of CO_2, and four molecules of ammonia (Figure 1.2) (Smith and Atkins, 2002; Todd et al., 2006). The released CO_2 can be re-fixed in photosynthesizing leaves and the resulting glyoxylate is metabolized by the photorespiratory pathway (Winkler et al., 1987; Todd et al., 2006).

1.2.1.3. Sub-cellular localization of ureide metabolism

Xanthine dehydrogenase (XDH) activity has been documented in peroxisomal preparations of pea (Corpas et al., 2008), however immunolocalization experiments in cowpea determined that the conversion of xanthine to urate occurs in the cytosol (Figure 1.3) (Datta et al., 1991). The oxidation of urate to allantoin occurs in the peroxisome, determined by the location of uricase (Van den Bosch and Newcomb, 1986; Hanks et al., 1981). Allantoinase and all subsequent enzymes in the ureide degradation pathway reside in the endoplasmic reticulum (Werner et al., 2008; Hanks et al., 1981; Serventi et al., 2010; Werner and Witte, 2011). It has yet to be determined how urate moves into the peroxisome and how allantoin is transported out of the peroxisome to the endoplasmic reticulum (Werner and Witte, 2011).

1.2.2. Abiotic stress

During the lifecycle of a plant, it encounters negative environmental constraints, or abiotic stresses, which can restrict the growth, reproduction or survival of the plant. Common abiotic stresses include drought, heat, cold, salinity, waterlogging, mineral deficiency, and mineral toxicity. Abiotic stresses cause extensive losses to agricultural production yields worldwide every year (Boyer, 1982).

Breeding resistance to abiotic stress in plants is challenging because abiotic stress resistance usually shows polygenic inheritance and is typically conditioned by multiple, interacting mechanisms (Miklas et al., 2006). It is also difficult to breed plants for abiotic stress resistance since plants respond differently to stresses depending on the frequency, duration, and intensity of the stress, as well as the growth stage of the plant (Muñoz-Perea et al., 2006).

1.2.2.1. Water limitation stress

Drought stress, or water limitation, is lack of water available to the plant, from precipitation and soil moisture, during its life cycle which constrains the full genetic potential of the plant (Beebe et al., 2013b). Drought has been predicted to be the most critical threat to world food security (Farooq et al., 2009). Photosynthesis and cell growth are among the primary processes to

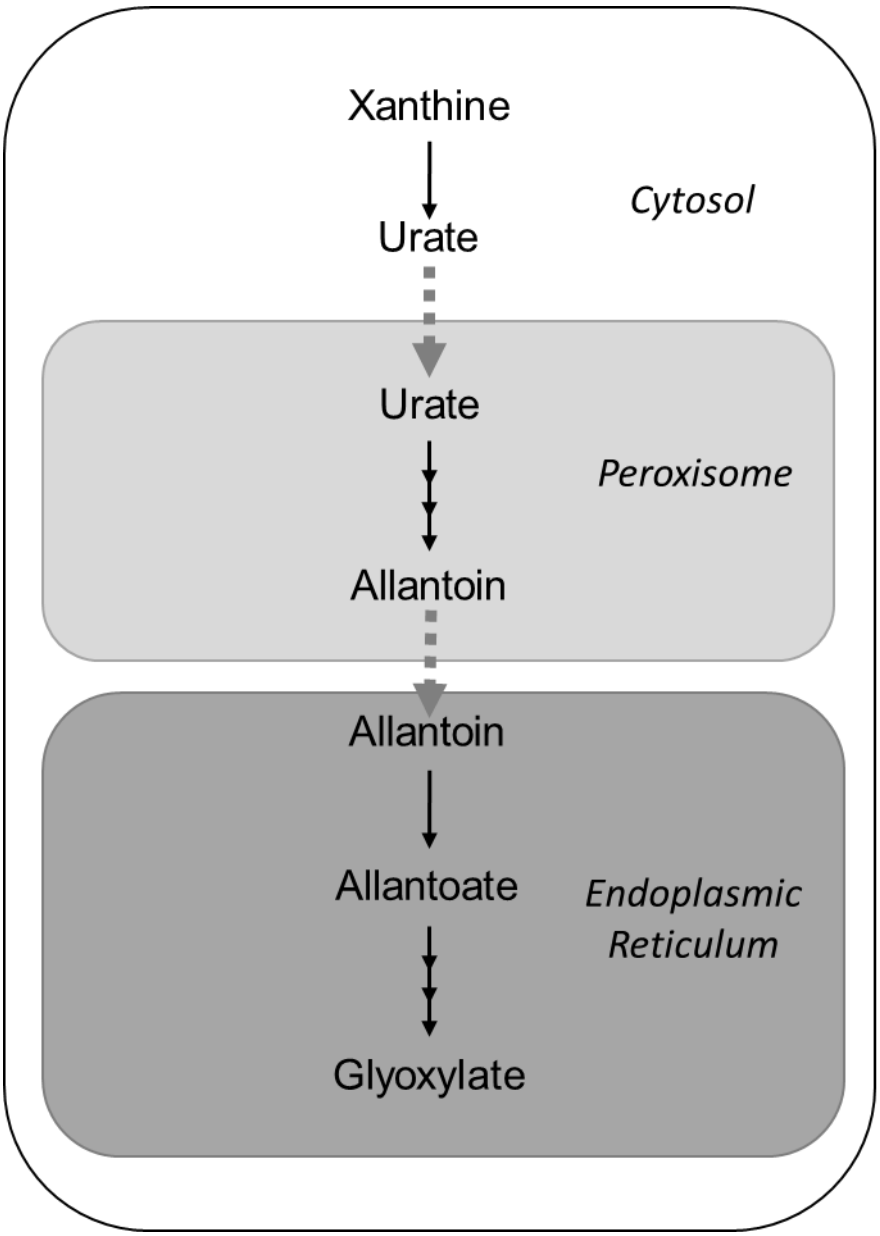


Figure 1.3: Cellular localization of ureide metabolism in plants. Adapted from Werner and Witte, (2011).

be affected by drought (Chaves, 1991). Early in the plant's response to drought, the stomata close in response to a decline in leaf turgor or water potential in an attempt to decrease evapotranspiration (Chaves et al., 2002). Eventually, the closure depresses gas exchange and reduces photosynthetic carbon assimilation (Flexas et al., 2004).

Under normal conditions, plants produce ROS mainly in the chloroplasts, the peroxisomes, and in the mitochondria (Cruz de Carvalho, 2008). If the concentration of ROS is kept low, it participates in stress signalling (Vranova et al., 2002). However, too much ROS becomes deleterious, initiating a cascade that oxidizes proteins, lipids, DNA and RNA (Cruz de Carvalho, 2008). This unrestricted oxidation of the cell membranes and other cellular components eventually leads to cell death (Dat et al., 2000). The production of ROS is intensified under drought stress and causes cellular damage (Chaves et al., 2009). ROS content is increased in several ways during drought stress, the greatest contributor being over-reduction of the photosynthetic electron transport chain (Noctor et al., 2002). Decreased availability of other oxidants for the electron transport chain may promote electron flow to dioxygen (O_2) and over-reduction of the chain enhances the likelihood of singlet oxygen generation at photosystem II (PSII) (Noctor et al., 2014). In the peroxisome, the majority of ROS produced during drought is due to accelerated activity of glycolate oxidase (produces H_2O_2) due to increased oxidation of ribulose-1,5- bisphosphate (RuBP) brought on by the combined effects of the decreased carbon dioxide (CO_2) concentration resulting from stomatal closure, and compounded by increased intracellular temperatures, leading to greater photorespiratory activity (Noctor et al., 2014).

1.2.2.2. Sub-zero temperature stress

Low temperature, chilling, and frost pose major limitations on agricultural plants. Crops from tropical origins grown in temperate climates, like bean and soybean, have been hypothesized to lack the genetic information needed to become frost tolerant (Allen and Ort, 2001). With the increased climatic variability predicted to result from climate change and the expansion of dry bean production to less ideal areas, research into making cultivars more resistant to abiotic stresses, including temperature stress, is important.

Plant species have optimum temperature ranges for growth and development. Below this temperature, plants exhibit signs of injury and delayed growth. Even more harmful to plants are

temperatures below freezing, in which ice formation, rather than just low temperatures, causes injury (Mahajan and Tuteja, 2005). The main sites of cold stress damage are the membrane systems. Damage can occur as physical damage from ice build-up or changes in lipid phase (Thomashow, 1999; Uemura et al., 1995). Most damage, including membrane damage, caused by chilling is due to the dehydration associated with freezing (Mahajan and Tuteja, 2005). Dehydration occurs due to the formation of ice in the extracellular space, which then draws intercellular water out of the cell due to the lower chemical potential of ice compared to water below 0°C (Olien and Smith, 1977; Charrier et al., 2015). Some plants can acclimate during low temperatures, which reduces the temperature which ice forms in the apoplastic space (Guy, 1990; Ouellet, 2007). Biochemical and physiological changes occur at whole plant and cell levels during acclimation, including a decrease in growth, a reduction of tissue water content, an increase in ABA content, adjustments in membrane lipid composition and an accumulation of compatible osmolytes (Joshi, 1999). However, no evidence of an ability to acclimate has been detected in bean or soybean (Allen and Ort, 2001).

Chilling stress in plants causes the generation of ROS and oxidative stress to occur (Prasad et al., 1994a; O'Kane et al., 1996). ROS is overproduced in the chloroplast by enhancing electron flux to O_2 and by causing over-reduction of the electron transport chain (Sharma et al., 2012). Chilling intensifies the imbalance between light absorption and light usage through the inhibition of carbon cycle activity. Lower temperatures reduce reaction rates and limit sinks for absorbed light energy, including CO_2 fixation and photorespiration (Huner et al., 1998), causing superoxide to form (Wise, 1995). ROS production during chilling also occurs in the mitochondria (Prasad et al., 1994b) and in the peroxisome (Suzuki and Mittler, 2006). Chilling injury has been proposed to be caused by oxidative damage due to the accumulation of ROS (Baek and Skinner, 2012). ROS production during cold stress causes lipid peroxidation, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death, and ultimately leads to the death of the cells (Larkindale and Knight, 2002; O'Kane et al., 1996; Sharma et al., 2012).

1.2.2.3. Induced oxidative stress

Chemical treatments can be used to induce ROS in a way similar to that which would occur during abiotic stress. Methyl viologen (MV) accepts electrons from the iron-sulfur cluster (Fe-S_A/Fe-S_B) of photosystem I and induces the production of superoxide (O_2^-) through the photoreduction of O_2 (Kim and Lee, 2003). The O_2^- is dismutated by superoxide dismutase, forming hydrogen peroxide (H_2O_2). The damage caused by MV is irreversible (Taylor et al., 2005). MV has been commonly used in stress response studies including studies of oxidative stress tolerance, function of antioxidant system components, ROS signaling, ROS induced cell death, and to mimic the drought effect on carbon and nitrogen metabolism (Lascano et al., 2007).

1.2.3. Ureide accumulation during abiotic stress

1.2.3.1. Ureide accumulation during drought

The relationship between drought and ureide accumulation was first documented when different legume species that were drought stressed accumulated ureides in the xylem sap (Sinclair and Serraj, 1995). The legume species that were ureide exporters (soybean, cowpea and common bean) exhibited greater drought-sensitive nitrogen fixation compared to the legumes that were amide exporters (chickpea, faba bean, lupine, pea, and peanut) (Sinclair and Serraj, 1995). Nitrogen fixation was the first physiological process to be affected by drought in ureide exporters, before the decrease in transpiration or stomatal closure (Vadez and Sinclair, 2001). A soybean genotype (Biloxi) that exhibits significant impairment of nitrogen fixation during drought accumulated more ureides in the petioles than a soybean genotype (Jackson) that demonstrates superior nitrogen fixation during drought (Serraj and Sinclair, 1996). Ureide accumulation during drought has been documented in petioles shoots, nodules, and leaves (de Silva et al., 1996; Serraj and Sinclair, 1996; Vadez et al., 2000; Vadez and Sinclair, 2001).

Ureide accumulation during drought conditions was previously considered to play a role in feedback inhibition of nitrogen fixation. It was hypothesized that leaf ureides increased, were transported to the nodules via the phloem, and inhibited the fixation in the nodules. Therefore, differences between cultivars in the sensitivity of nitrogen fixation to water limitation were believed to be due to differences in leaf ureide metabolism (King and Purcell, 2001; Sinclair et al.,

2003; Purcell et al., 2000). This theory was challenged when soybean that were watered after a period of drought reinitiated nitrogen fixation before levels of leaf ureides decreased (King and Purcell, 2005). Also, when the early stages of drought response were studied in nitrogen-fixing soybean, the inhibition of fixation occurred concurrently with the accumulation of ureides in the nodules but before the accumulation of ureides in the shoots was observed (Ladrera et al., 2007). Although ureides accumulate in the nodules, there are no data to support that ureide accumulation is the mechanism responsible for inhibiting nitrogen fixation (Ladrera et al., 2007).

The relationship between abiotic stress and ureide accumulation has predominately been documented in legume species that are actively fixing nitrogen. However, ureides have been measured in non-fixing legumes during normal physiological states (Werner et al., 2013), and the quantification of ureides in non-fixing legume species during abiotic stress has also been noted. Alamillo et al. (2010) observed an increase in ureides in nitrate-fertilized *Phaseolus vulgaris* under conditions of drought stress in the roots, shoots and leaves. Ureide accumulation has predominately been studied during conditions of water limitation, however, in Arabidopsis, allantoin has been also shown to accumulate at low levels under extended periods of darkness and increased leaf age (Brychkova et al., 2008). Ureides also accumulate in *Salicornia persica* and *Sarcocornia fructicosa* under increasing salt concentrations provided in irrigation solutions (Ventura et al., 2011) and *Eutrema salsugineum* under growth in high light (Malik et al., 2015). Therefore, it is likely ureide accumulation occurs in response to general abiotic stress.

Ureides have also been measured following cold stress. Strauss et al. (2007) quantified ureides in leaves of two cultivars of soybean: one chilling tolerant and one chilling sensitive. They saw a decrease in leaf ureide content following a cold treatment of 6°C overnight for 12 consecutive nights (light period temperature of 26°C). The decrease was greater when the whole plant was chilled, compared to chilling the shoots only, and was greater in the chilling sensitive cultivar (Strauss et al., 2007). These authors used nitrogen-fixing soybean plants, therefore, the reduction in ureides could have been due to a decrease of ureides being transported during nitrogen fixation. Under cold soil temperatures induction of nitrogen fixation is delayed, nitrogenase activity decreases and at a low enough temperature (below 10ºC) nodulation ceases (Zhang et al., 1995). Ureides move by transpiration flow (Baral et al., 2016), however, chilling causes shoot dehydration due to the altered balance of root water uptake and leaf transpiration (Sanders and Markhart III, 2001; Vernieri et al., 2001). If ureide production decreases and movement through

the shoot is diminished, the decrease in nitrogen fixed ureide content may mask the increase of ureides *in situ* accumulating due to the cold stress.

1.2.3.2. Regulation of ureide metabolism during drought stress

Allantoinase (*ALN*) gene expression was determined to increase in bean under conditions of drought stress and after treatment with ABA (Alamillo et al., 2010). Since then, a second *ALN* gene was identified in bean and four *ALN* genes were identified in soybean, with two of the soybean genes (*GmALN1* and *GmALN2*) being abundant in all tissues and the other two (*GmALN3* and *GmALN4*) expressed primarily in the nodules (Duran and Todd, 2012). ALN activity increased in bean in the shoots and roots during drought stress, but not in the leaves (Alamillo et al., 2010).

Allantoate amidohydrolase (AAH) was first identified in plants from Arabidopsis and was shown to be expressed in every tissue examined (seed, seedling, leaf, root, stem, flower and silique) at several developmental stages (Todd and Polacco, 2006). During drought stress, *AAH* expression does not change in soybean leaves, therefore it was hypothesized post-translational modifications were involved in AAH activity (Charlson et al., 2009). However, since AAH activity also does not change during drought in soybean leaves (M.Munson, personal comm.), it is assumed AAH does not play a role in the ureide drought response in soybean. In bean, *AAH* expression in the leaves does increase slightly during drought treatment (Alamillo et al., 2010), however in another study bean AAH activity was shown to decrease (Díaz-Leal et al., 2014). Therefore, timing and species differences may exist in regulation of AAH under drought stress.

Little research into the enzymes upstream and downstream of ALN and AAH during drought stress has been completed. However, in soybean and bean uric oxidase activity has been shown to decrease in the nodules but not in the roots or shoots in water-limited plants (Gil-Quintana et al., 2013; Alamillo et al., 2010).

1.2.3.3. Evidence ROS induces ureide accumulation

Why ureides accumulate in plants undergoing water limitation is still in question. One possible answer is that ureides accumulate from recycling of existing purines because of senescence. Alternatively, ureides may be produced to decrease the effects of the stress on the plant. One theory that has been postulated is that ureides accumulate to scavenge ROS (Brychkova et al., 2008).

Xanthine oxidoreductase converts hypoxanthine to xanthine and xanthine to uric acid. It exists in two interconvertible forms: xanthine dehydrogenase (uses NAD^+ as electron acceptor; Equation 1.1) and xanthine oxidase (uses O_2 as electron acceptor; Equation 1.2) (Corpas et al., 2008; Werner and Witte, 2011). The form xanthine dehydrogenase takes is species specific, from no oxidase activity in wheat (Montalbini, 1998) to predominant oxidase activity in pea leaves (Corpas et al., 2008). The oxidase form of the enzyme produces O_2^- (Yesbergenova et al., 2005) which can be converted to H_2O_2 by the enzyme superoxide dismutase (McCord and Fridovich, 1969). XDH knockdown lines have diminished O_2^- content but increased levels of H_2O_2 compared with wildtype during drought stress, which suggests XDH mutants have reduced capacity to detoxify ROS (Watanabe et al., 2010). The accumulation of H_2O_2 was diminished by the addition of urate, allantoin or allantoate, when added exogenously (Werner and Witte, 2011; Watanabe et al., 2010).

Equation 1.1: $$Xanthine + H_2O + NAD^+ \rightarrow Urate + NADH + H^+ \quad [1.1]$$

or, under depleted NAD^+

Equation 1.2: $$Xanthine + H_2O + 2O_2 \rightarrow Urate + 2\,O_2^- \quad [1.2]$$

The conversion of urate to allantoin by uricase also yields H_2O_2 (the net conversion of urate to allantoin, including the activity of HIU hydrolase and OCHU decarboxylase, is depicted in equation 1.3) (Angermuller and Fahimi, 1986; Ramazzina et al., 2006). As the allantoin produced by the conversion likely comes into contact with the produced H_2O_2, allantoin also may scavenge the H_2O_2 produced by upstream enzyme activity (Werner and Witte, 2011).

Equation 1.3: $Urate + O_2 + 2H_2O + H^+ \rightarrow Allantoin + H_2O_2 + CO_2$ [1.3]

Arabidopsis plants with a mutation in the gene encoding XDH (*Atxdh* mutants) had greater chlorophyll degradation than wild type (Col-0) plants after 6 days of continual dark treatment, which was intended to induce metabolite remobilization processes. Addition of 0.1 mM allantoin or allantoate alleviated the damage (Brychkova et al., 2008). Since chlorophyll degradation is linked to ROS accumulation, Brychkova et al. (2008) suggested that the accumulation of allantoin may occur to scavenge ROS produced by the stress. They observed that application of allantoin and allantoate attenuated leaf mortality caused by the addition of H_2O_2 (Brychkova et al., 2008). Whether the exogenous allantoin and allantoate directly scavenged the ROS, or indirectly attenuated the ROS production, was not determined.

1.2.3.3.1. Subcellular location of ROS generating activities

In order for ureides to scavenge ROS directly they must come into contact with the ROS. As previously discussed (see section 1.2.1.3), uric acid is transported from the cytosol to the peroxisome and allantoin moves from the peroxisome through the cytosol to the endoplasmic reticulum (ER) (Figure 1.3). Allantoate, ureidoglycine, and ureidoglycolate all reside in the ER (Werner and Witte, 2011). The major sources of ROS production in plants are those organelles with a highly oxidizing metabolic activity, with an intense rate of electron flow, namely the chloroplast, the mitochondria and the peroxisome (Tripathy and Oelmüller, 2012). During normal metabolism, peroxisomes, mitochondria, and chloroplasts produce superoxide (Rio et al., 2003). H_2O_2 is produced during normal metabolism due to the presence of oxidases in the peroxisome, including but not limited to acyl-COA oxidase, employed in fatty acid oxidation, and glycolate oxidase, a photorespiratory pathway enzyme (Van den Bosch et al., 1992; Hu et al., 2012). The accumulation of ROS is also triggered by environmental stresses (Tripathy and Oelmüller, 2012). During stress, stomatal closure causes limitation of CO_2 uptake and favors photorespiratory production of H_2O_2 in the peroxisome and the production of ROS, due to the over-reduced photosynthetic electron transport chain in the chloroplast (You and Chan, 2015; Gill and Tuteja,

2010). To a lesser extent, ROS is also produced in the cell walls and the plasma membrane, usually in response to stress signalling (Tripathy and Oelmüller, 2012).

ROS production in the ER is starting to gain attention. Significant peroxide accumulation in the animal ER is caused by oxidation of cysteine residues during disulphide bond formation (Harding et al., 2003; Tu and Weissman, 2004). Under abiotic stress, the demand for protein folding can exceed folding capacity and lead to an accumulation of unfolded or misfolded proteins, causing ER stress. ER stress leads to the production of H_2O_2 (as well as lipid peroxidation and protein oxidation) in Arabidopsis, but this H_2O_2 is mainly thought to trigger ROS signaling (Ozgur et al., 2014).

While ureides do not come in direct contact with ROS in the chloroplast or mitochondria, uric acid and allantoin may come into contact with the ROS produced in the peroxisome or the ROS that have moved into the cytosol. Allantoin, along with the downstream ureides, may also come into contact with ROS produced in the ER.

1.2.3.3.2. Medical implication of ureide metabolism in humans

Although the concept of ureides acting as ROS scavengers may be a relatively new idea in plant biology, it has been hypothesized in humans for over 30 years (Ames et al., 1981). Ames et al. (1981) hypothesized that urate scavenged singlet oxygen, *in vitro*, by observing the decrease of urate after treatment with superoxides. Urate is also oxidized by hydrogen peroxide in the presence of hematin or methemoglobin (Ames et al., 1981; Howell and Wyngaarden, 1960).

During primate evolution, a mutation caused the inactivation of uricase, the enzyme that catalyzes the conversion of uric acid to allantoin (Oda et al., 2002). The HIU hydrolase gene also has several inactivating mutations but, interestingly, the OHCU decarboxylase gene potentially codes for a functional protein but has not yet been shown to be expressed (Ramazzina et al., 2006). It is hypothesized that an abrupt evolutionary event that led to the accumulation of uric acid is a consequence of the capacity of uric acid to inhibit the leakage of inflammatory cells caused by peroxynitrite (the product of free radicals of nitric oxide and superoxide) and therefore prevent oxidative damage to increasingly complex brains (Scott and Hooper, 2001). Howell and Wyngaarden (1960) determined the product of peroxidation of uric acid was allantoin, therefore any allantoin in humans could be assumed to be from oxidative conversion of urate. Therefore, it

was suggested allantoin concentration could be used as a marker of free-radical reactions taking place in the body (Grootveld and Halliwell, 1987). Since then, measuring allantoin concentration has been used to serve as a biomarker for oxidative stress. Allantoin increases in the plasma of heavy smokers, chemotherapy patients, and following neonatal hypoxia during labour (summarized in Czerska et al., 2015). Allantoin accumulation has been documented in patients with rheumatoid arthritis (Yardim-Akaydin et al., 2004), patients with complications from dialysis (Kand'ár et al., 2006), type 2 diabetics (Chung and Benzie, 2013), and following completion of vigorous exercise (Hellsten et al., 1997). The research in humans, combined with the observation that ureide levels increase in plant tissue undergoing abiotic stress, raises the question of whether allantoin accumulation can serve as a marker of oxidative stress in other organisms, even with the added complication of the presence of active uricase and ureide catabolic pathway. Allantoin also may be beneficial to humans. When applied topically, allantoin is believed to help protect DNA from ultraviolet radiation and is also used for its healing, soothing and anti-irritating properties (Araujo et al., 2010; Korać and Khambholja, 2011), however more research needs to be completed to confirm these roles.

1.2.3.3.3. Does ureide metabolism respond to stress?

Despite a role for uric acid in scavenging ROS, the question of the antioxidant potential of allantoin remains. Conflicting conclusions have been drawn regarding this question. Gus'kov et al. (2001) suggest allantoin is an antioxidant. They came to this conclusion after observing mice with stimulated oxidative stress, generated using a hyperbaric chamber, with and without intraperitoneal injections of allantoin. Mice injected with allantoin have decreased lipid peroxidation in erythrocytes, brain, lungs, liver, and kidney (Gus'kov et al., 2001). Malonic dialdehyde (MDA) levels are lower in the stressed mice injected with allantoin than in the control mice not subjected to oxidative stress (Gus'kov et al., 2001). Later, this group assessed the effect of allantoin on mutagenesis induced by H_2O_2 in *Escherichia coli*. Allantoin, added to the bacterial suspension before treatment with H_2O_2, decreased the mutation frequency compared to *E.coli* without exogenous allantoin (Gus'kov et al., 2002). They also confirmed that treatment with allantoin, at concentrations between 10^{-3} M and 10^{-6} M, prior to addition of H_2O_2 decreases the

frequency of chromosomal aberrations caused by H_2O_2 in onion seedlings (Gus'kov et al., 2004). Although their data support allantoin having positive effects during stress, this research does not demonstrate that allantoin itself directly interacts with any specific ROS.

More recently the antioxidant role for allantoin has been questioned. Although exogenous allantoin stimulates plant growth, increasing soluble sugars and free proline content in rice seedlings, allantoin does not increase free radical-scavenging capacity, reducing power, linoleic acid peroxidation inhibition, or chelating activity (Wang et al., 2012). In contrast with vitamin C, which increases antioxidant activity with increasing concentration, increasing concentrations of allantoin have no effect (Wang et al., 2012). Since all of these tests were done *in vitro*, studies to determine whether allantoin can function as an efficient antioxidant *in vivo* need to be completed (Watanabe et al., 2013).

It is possible that along with, or instead of, scavenging ROS, the accumulation of ureides during abiotic stress plays alternate stress mitigating roles. Molecules that mitigate abiotic stress damage can play a number of different roles, including, but not limited to: signalling (Alcázar et al., 2011); stabilization of macromolecules, such as proteins, lipids or DNA (Bachrach, 2005); or acting as compatible osmolytes to retain cellular hydration (Zhu, 2002). To date, with the exception of influencing ABA signaling, none of these possibilities has been extensively researched as a role of ureides during stress. Elevated allantoin concentrations, caused by either an *aln* mutation, or exogenously added allantoin, activates ABA production in Arabidopsis (Watanabe et al., 2013). ABA activation is regulated both transcriptionally and post-translationally. Transcriptionally, allantoin activates transcription of an ABA biosynthesis enzyme, *NCED3*. Post-translationally, allantoin activates a β-glucosidase responsible for hydrolysing glucose-conjugated ABA called BG1 (Watanabe et al., 2013). The same group observed that genes induced by ABA, drought stress and osmotic stress showed upregulated expression in wild type Arabidopsis under addition of allantoin, in a dose-dependent manner (Watanabe et al., 2013). As ABA functions in integrating various stress signals and controlling stress responses (Tuteja, 2007), allantoin may function in activating signalling during stress.

1.2.4. Ureide accumulation under field conditions

Ureides have been measured in tropical legumes in the field since the 1980s, however the purpose of these tests was not to determine stress levels. Rather, ureides were used to determine the rate of nitrogen fixation. After preliminary analysis of ureide content of plants in the greenhouse (Herridge, 1984), methods were developed for field study (Herridge et al., 1988). Stem extracts and xylem sap are used to calculate the relative ureide abundance by comparing it to the total nitrogen in the sample (Bergersen et al., 1989). These provide statistically significant correlations between relative ureide quantity and crop dry matter, nodule weight, and estimation of plant available soil nitrogen ($\delta^{15}N$ r=-0.92) (Herridge et al., 1990). Although the information gained from this procedure was informative to the analysis of nitrogen fixation rates, an updated understanding of ureides that accumulate in plant tissue for reasons other than increased nitrogen fixation needs to be addressed.

A comparison of ureide accumulation during drought stress between two soybean genotypes was completed in the field by Purcell et al. (1998). They found soybean cultivars Jackson and Biloxi accumulated ureides in the petioles under water stress, but Biloxi ureide petiole content was higher (Purcell et al., 1998). Ureide accumulation was also quantified in leaves and petioles of a third field grown soybean cultivar (KS4895) and compared to Jackson (Purcell et al., 2000). Large field experiments with many genotypes are necessary to determine whether the relationships seen in the growth chamber and greenhouse occur outside of a controlled environment and whether ureide accumulation can be correlated to abiotic stress tolerance of a genotype.

1.2.5. Soybean, common bean, and tepary bean

In addressing my hypotheses, I utilize three ureide-exporting legume species: *Glycine max* (soybean), *Phaseolus vulgaris* (common bean), and *Phaseolus acutifolius* (tepary bean). These three species belong to the phaseoloid-millettioid clade (which contains the genera *Glycine*, *Phaseolus*, and *Vigna*) (Shoemaker et al., 2006). Soybean and common bean diverged ~19 million years ago (McClean et al., 2010). Although synteny is only found in short blocks in these genomes, a high degree of sequence homology has been reported to be shared between soybean and common bean (Boutin et al., 1995). Tepary bean diverged from the rest of the Phaseoli approximately 2.5

million years ago (Delgado-Salinas et al., 2006; Porch et al., 2013). Tepary bean and common bean are highly syntenic with only a few intra-chromosomal rearrangements (Gujaria-Verma et al., 2016). Experiments focussing on ureide metabolism were completed using soybean, similar to much of the research done thus far. The experiments completed in the field trials focussed on the two *Phaseolus* species as the intention is to contribute to a knowledge base that will assist in increasing abiotic stress tolerance in common bean.

1.2.5.1. Soybean and abiotic stress

Soybean, *Glycine max* (L.) Merr., is one of the most valuable crops in the world, due in part to its wide range of end uses, including oil products, livestock and aquaculture feed, human foodstuff and biofuel. Demand for soybean has been rapidly increasing and, although yield has been increasing, most of this demand has been met by increased production (Masuda and Goldsmith, 2009). As world acreage is limited, increasing yield of soybean is necessary to keep up with global demand. One way to facilitate a growth in average yield is to increase soybean's tolerance to abiotic stress.

Worldwide, soybean is only yielding 60-80% of its potential. The gap can be attributed, in large part, to suboptimal environmental conditions for growth (Board and Kahlon, 2011). The most damaging abiotic stress on soybean production is drought stress (Kulcheski et al., 2011). Soybeans are so sensitive to water stress that solely removing irrigation for the last three weeks of the growing period can reduce yields by 65% (Eck et al., 1987).

Most papilionoids, soybean's subfamily, are diploids (Shoemaker et al., 2006). However, the genome of soybean has undergone a full duplication (Shoemaker et al., 1996) and is classified as a partially diploidized tetraploid (Shultz et al., 2006). Therefore, when studying the genome of soybean, it is usual that there are twice as many genes encoding the same enzyme as in other legumes. As an example, this is the case in ureide metabolism. Soybean contains four allantoinase genes compared to two in common bean (Duran and Todd, 2012).

1.2.5.2. Common bean and abiotic stress

Common bean, *Phaseolus vulgaris* L., is a widely produced agricultural crop and is the most important grain legume for human consumption (Kelly, 2010; Schmutz et al., 2014). Beans play a significant role in diets of around the world, specifically in developing countries (Messina, 1999). However, consumption of beans is income-inelastic: consumption drops as economic levels rise (Broughton et al., 2003). Beans are high in protein, fiber, vitamins, and minerals and have been linked with having a preventative effect against diseases such as cancer, diabetes and heart disease (Anderson et al., 1999), therefore, increasing global consumption would be beneficial.

The majority of the world's bean production happens in low input agricultural systems of developing countries (Miklas et al., 2006) and much of the world's production of bean is being relocated to marginal land due to acreage competition from other crops such as soybean and maize (Beebe et al., 2013a). As production shifts, these plants become more vulnerable to environmental stressors; enhancing the abiotic stress tolerance in common bean becomes more important and developing cultivars with increased stress tolerance is the primary goal of bean breading programs worldwide (Miklas et al., 2006). Cultivated common beans lack resistance to many abiotic stresses. For the purpose of this thesis, I will focus on two important abiotic stresses affecting common bean: water limitation stress and sub-zero temperature stress.

Water limitation, or drought stress, affects over 60% of dry bean production (Rao et al., 2013). Due to its importance, breeding for drought resistance has a long history, mainly in Latin America. More recently, drought nurseries have also been developed in Africa (Beebe et al., 2013b). Bean production is greatly affected by both terminal and intermittent drought, therefore much of the breeding done thus far to increase tolerance to both have focussed on the traits of earliness and partitioning toward reproductive structures (Beaver et al., 2003). As such, many areas of improvement are left to be explored.

Much of the cold stress research done in bean has focussed on germination at low temperatures. Common bean has limited germination below 15°C (Dickson and Boettger, 1984), which greatly limits the number of frost free days in the production cycle in many areas. However, sub-zero temperature stress after germination is another important area for improvement. A sub-zero temperature stress at the seedling stage is very damaging to bean crops. In a study of ten species of legumes, pinto beans and navy beans were the least tolerant to freezing temperatures at the seedling stage (Meyers and Badaruddin, 2001). Meyers et al. (2001) also found that the

unifoliate leaf and the first trifoliate leaf stages were the most sensitive (Meyers and Badaruddin, 2001). Their research concluded bean had an LD_{50} of -3.25°C although regrowth after survival was even lower (Meyers and Badaruddin, 2001). Ashworth (1985) puts this number even higher, at -2.7°C, with the first nucleation in bean occurring at -1.3°C (Ashworth et al., 1985). An increase in tolerance of only 2-3°C would allow for expansion of dry bean production into areas with relatively shorter growing seasons, including the Canadian prairies and the higher altitudes of the tropics (Balasubramanian et al., 2004a).

1.2.5.3. Tepary bean and abiotic stress

Phaseolus acutifolius Gray is a wild relative of common bean. *P. acutifolius*, known as tepary bean, and was first identified in Northwestern Mexico and Southern Arizona (Nabhan and Felger, 1978; Kelly, 2010). Tepary bean is used as a subsistence crop in areas of the Southern US and Northern Mexico, however little research has been done using *P. acutifolius* in large-scale breeding programs. However, because it has high protein content, high productivity, adaptation to arid environments, and a wide profile of disease resistance, research on tepary bean is warranted, including its use as a donor parent to introduce new genes into a related plant (Nabhan and Felger, 1978). Interspecific crosses between common bean and tepary bean have been accomplished to transfer Common Bacterial Blight (CBB) resistance genes from tepary bean to common bean (Thomas and Waines, 1984). Since tepary bean has many other beneficial traits, including abiotic stress resistance, crossing to try to transfer abiotic stress resistance genes to common bean would be useful. Because only a small portion of genetic variability in tepary bean has been utilized for common bean improvement, there are still large gains that can be made through interspecific gene transfer (Singh, 2001).

Tepary bean is more resistant to many stresses than common beans, including high temperature stress (Gaur et al., 2015), sub-zero temperature stress (Balasubramanian et al., 2004a; Martinez Rojo, 2010), salt stress (Goertz and Coons, 1991), and drought stress (Beebe et al., 2013b). Again, for the purpose of this thesis, I will focus on water limitation stress and sub-zero temperature stress.

Most of the research completed on abiotic stress tolerance or avoidance in tepary bean has been completed on drought, or water limitation, stress. Tepary beans can withstand drought conditions better than common bean (Thomas et al., 1983). It is believed this is due, at least in part, to the deeper rooting systems and quicker closing stomates (Markhart III, 1985), thereby postponing dehydration. Differences in the response to drought occur within tepary bean accessions (Mohamed et al., 2002).

Although tepary bean germplasm has not been widely studied for genotypic differences to sub-zero temperature stress, there are reasons to believe that tepary beans have greater tolerate to sub-zero temperature stress than common beans. This tolerance is not apparent at germination, as tepary beans take longer to germinate at lower temperatures compared to common bean (Scully and Waines, 1987), but is apparent at the seedling stage. Balasubramanian (2004) observed a tepary genotype (PI535248) having an LD_{50} (50% of the leaflets were killed) 0.5°C lower than any of the common beans studied in the field and at least a 1.0°C lower LD_{50} avoidance in a controlled environment (Balasubramanian et al., 2004a). Future experiments by Martinez-Rojo (2007) observed two other tepary lines (PI 219445 and W6 15578) to have tolerance greater than that of the common beans studied, one which (NY5-161) had been previously categorized as tolerant to the cold (Martinez-Rojo et al., 2007).

1.2.5.4. Interspecific introgression lines of common bean and tepary bean

Bottlenecks that occurred during species domestication reduced variability in plant populations. The use of wild relatives in breeding programs has been beneficial as they provide a broad pool of potentially useful genetic resources. To date, wild relatives in a variety of breeding programs have been used to improve pest resistance, disease resistance, abiotic stress tolerance, yield, and quality traits along with providing cytoplasmic male sterility or fertility restorers for hybrid crops (Hajjar and Hodgkin, 2007).

Bean has been widely studied, but still has limited growth in many locations due to climatic constraints. Because of previous intensive research, modern production has a restricted genetic base and other tactics have to be employed to gain genetic variability in order to increase tolerance to problems like abiotic stress (Kelly, 2010; Nabhan and Felger, 1978). Untapped crop wild

relatives, like tepary bean, and closely related species need to be explored to understand any novel genetic variation which may assist increasing abiotic stress tolerance in bean (Porch et al., 2013).

P. acutifolius is part of *P. vulgaris'* tertiary gene pool, meaning crosses between the two require embryo rescue and one or more backcrosses are required to restore fertility (Singh, 2001). Usually, to avoid nuclear-cytoplasmic interactions, common bean is used as the female parent. If nuclear cytoplasmic interactions are absent, the use of tepary bean as the female typically results in a higher frequency of usable embryos, however, data on reciprocal crosses is often conflicting due to the different genotypes used (Waines et al., 1988). Another problematic situation is caused by an incompatibility locus that occurs in some *P. vulgaris* genotypes (Parker and Michaels, 1986).

1.2.6. Ureides in common bean and tepary bean

Glycine and *Phaseolus* diverged from a common ancestor ~19 million years ago but still have extensive blocks of shared loci (Shoemaker et al., 2006; McClean et al., 2010; Kelly, 2010). Common bean and soybean are similar in that they both transport fixed nitrogen as ureides (Schubert, 1986). Common bean has also been used in studies of ureide accumulation during conditions of abiotic stress (ie: Alamillo et al., 2010). It is reasonable to assume that soybean and common bean share common regulatory mechanisms with respect to ureides and abiotic stress.

No mechanistic studies of ureide metabolism have been completed using *P. acutifolius*, however ureides have been measured in tepary bean to determine nitrogen fixation differences between accessions (Crews et al., 2004). Tepary beans have not been examined for changes to ureide content during abiotic stress. One of the goals of this thesis was to investigate this relationship.

1.3. Objectives

This thesis explores ureide accumulation during abiotic stresses, specifically water limitation, sub-zero temperature stress and induced oxidative stress. The first part of the thesis addresses the mechanism of ureide accumulation, testing whether there is a direct link between ureide accumulation and ROS. The hypothesis that ureides accumulate during water limitation in nitrogen-fertilized soybean in a manner similar to nitrogen-fixing soybean was tested. Also, I tested the hypothesis that ureide accumulation occurs in response to an increase of ROS, and that ureides scavenge the excess ROS produced during abiotic stress. A third hypothesis I tested is that increased ureide content has a beneficial effect on cellular integrity and will mitigate damage in response to ROS.

The second part of the thesis documents a series of experiments designed to determine if ureide accumulation can act as a tool to determine abiotic stress tolerance of a genotype. I pursued this objective by studying the correlation between ureide accumulation and plant productivity or survival under stress. I hypothesized that ureide accumulation under sub-zero temperature stress and water limitation differs between genotypes in a population and the variation in ureide accumulation correlates with the differences in abiotic stress tolerance among the genotypes. I also wanted to test the hypothesis that stress tolerance genes can be introgressed from tepary bean into common bean by analyzing stress response of a previously developed population of interspecific introgression lines of tepary bean and common bean.

Specifically, the objectives of my research were:

i. To quantify ureide accumulation under drought stress in both nitrogen-fixing and nitrogen-fertilized tissue and determine changes in activity of a key enzyme, allantoinase, during this stress.
ii. To establish if ureide accumulation can be triggered by an increase in ROS, using a ROS inducer, methyl viologen.
iii. To determine if increased ureide content decreases cell damage in response to ROS.
iv. To determine if differences in ureide accumulation exist in a population of tepary bean under oxidative stress.

v. To determine if interspecific introgression lines of common bean and tepary bean exhibit greater tolerance to water limitation and sub-zero temperature stress than the common bean parent.

vi. To quantify ureide accumulation during sub-zero temperature stress and water limitation stress in field grown tepary beans, common beans and interspecific introgression lines and to determine if the response of ureide content changes to sub-zero temperature stress differ between these groups.

vii. To establish whether the buildup of ureides during abiotic stress is correlated with stress tolerance under abiotic stress conditions in the field in a population of tepary bean, common bean and interspecific introgression lines and to assess the utility of measuring ureides as a breeding tool for selection of abiotic stress tolerant genotypes.

In accomplishing these objectives, I hoped to contribute to an increased understanding of the mechanism(s) that influence the relationship between ureide metabolism and abiotic stress in legumes and other plants.

2. Material and Methods

2.1. Soybean growth conditions

'Williams 82' soybean seeds (*Glycine max L.* cv. Williams 82') were acquired from Missouri Foundation Seed (Columbia, MO, USA). Seeds were germinated in 10″x15″ water-saturated germination paper (Anchor Paper, St.Paul, MN, USA) at 23/18ºC day/night temperature, 16 hour photoperiod. After 4 days, germinated seedlings were transferred into 8″ pots containing 1 kg of 1:1 (v:v) mix of Sunshine Mix 1 - vermiculite and perlite (Sun Gro Horticulture Canada Ltd., Seba Beach, AB) and potting soil (Early's Farm and Garden Center, Saskatoon, SK). Soybean seedlings intended to fix nitrogen were inoculated by addition of peat containing *Bradyrhizobium* japonicum (Turno-N, Semences Pro-grain Inc., QC, Canada).

2.1.1. Controlled environment experiments

Plants were grown at 25°C/23°C (day/night temperature) at 50-60% relative humidity with a 16 hour photoperiod and watered to 80% pot capacity (PC) (determined by weight) every second day until the treatment period. Seedlings were thinned to 1 per pot 14 days post planting. Plants were fertilized every 10 days with nutrient solution containing 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.312 mM K_2SO_4, 0.5 mM K_2HPO_4 and 25 mM $FeSO_4$/Na_2EDTA, 2.5 mM H_3BO_3, 1 mM $ZnSO_4$, 0.1 mM $NaMoO_4$, 0.11 mM $NiCl_2$, 0.15 mM $CuSO_4$, 0.01 mM $CoCl_2$, and 2 mM $MnSO_4$. Nutrient solution supplied to non-inoculated seedlings also included 5 mM NH_4NO_3. Experimental treatments began at 40-42 days post planting (V4-V5 stage). Presence or absence of nodules was confirmed visually on disposal of the plants after the experimentation. The tissue from plants that deviated from the intended nitrogen source was discarded.

2.1.1.1. Water limitation treatment

Control plants were watered to 80% PC daily. The soil of water-limited (treated) plants was allowed to dry to 20% PC, and maintained by watering for nine days, then irrigation was completely discontinued. Two leaf disks (1.77 cm^2) of fully expanded new leaves from three plants were taken (excluding the mid-rib), pooled and stored at -80°C for further analysis. Sampling occurred every two days until plants fully senesced (approximately day twelve). This procedure was repeated at least three times (biological replicates).

2.1.1.2. Methyl viologen treatment

Methyl viologen (MV) (0.5 mM) was sponged onto the adaxial side of four or five fully expanded new leaves. Samples were taken at 0, 6, 12, 24, 36, and 48 hours after MV treatment. Two leaf disks (1.77 cm^2) taken from three separate plants undergoing the same treatment were combined and stored at -80°C for further analysis. This procedure was repeated at least three times (biological replicates).

2.1.2. Isolated leaf disks experiment

2.1.2.1. Preliminary experiment conditions

Soybean plants were grown at the conditions described above (see section 2.1 and 2.1.1). Three leaf disks (1.77 cm^2) were punched from fully expanded young leaves of nitrogen-fertilized and nitrogen-fixing soybean. Leaves were placed in 5 mL beakers containing 3 mL of water, 0.5 mM MV, a MV and allantoin solution (0.5 mM MV and 1mM allantoin), or a MV and uric acid solution (0.5 mM MV and 1 mM uric acid). After 24 hours and 48 hours with lights constantly on (approximately average of 4.7 $\mu mol\ s^{-1}\ m^{-2}$), leaves and solution were transferred to 15 mL conical tubes and cell death was analyzed by an electrolyte leakage assay (see section 3.3.5).

2.1.2.2. Treatment conditions

Soybean plants were grown as described above (see section 2.1 and 2.1.1). Leaf disks (1.77 cm^2) were punched from fully expanded young leaves from 40-45 day old soybean plants. Leaf

disks were submerged in 5 mL of either water, 1 mM allantoin or 1 mM uric acid. After 24 hours in a fume hood with constant fluorescent light (average of 4.7 µmol s^{-1} m^{-2}), either hydrogen peroxide was added to a final concentration of 40 mM, or MV was added for a final concentration of 0.5 mM, and samples were left for an additional 24 hours. Four leaf disks per treatment were used for HPLC analysis of ureide content, ROS assays, lipid peroxidation assays, or RNA extraction for RT-PCR. Electrolyte leakage assays were performed on tissue treated similar to above, but three leaf disks were submerged in 3 mL of either water, 1 mM allantoin, or 1 mM of uric acid, and then treated similarly after addition of either of hydrogen peroxide or MV.

2.2. Common bean, tepary bean and common x tepary interspecific introgression lines

2.2.1. Population relationships

A population consisting of common bean, tepary bean and interspecific introgression lines (common bean x tepary bean; BC_2F_6 and greater) donated by K. Bett were analyzed in the field trials and in laboratory analysis. NY5-161 was the common bean parent and a wild tepary, W6 15578, was the tepary bean parent (Figure 2.1). Both parents were classified as more tolerant to the cold than other accessions of their species (Martinez-Rojo et al., 2007). An F_1 population had been generated out of a cross using NY5-161 as the female. The resulting embryos required embryo rescue to survive. The ensuing plant had been backcrossed with NY5-161, yielding 3 BC_1 offspring from embryo rescue. Each of the three BC_1 had again been backcrossed to NY5-161 and resulted in 6 BC_2 individuals. In this study, I used accessions of at least BC_2F_6 to ensure homozygosity (Martinez Rojo, 2010 and V. Gurusamy personal comm.). The specific numbers of genotypes used in the trials are indicated in the method and results of each experiment.

2.2.2. Tepary bean population survey

2.2.2.1. Growth conditions

A germplasm survey study was conducted on 118 tepary bean genotypes provided to me as one month old plants. After this initial experiment of ureide accumulation, a subsample of 29 genotypes was selected for replication. These 29 genotypes covered all different observations seen

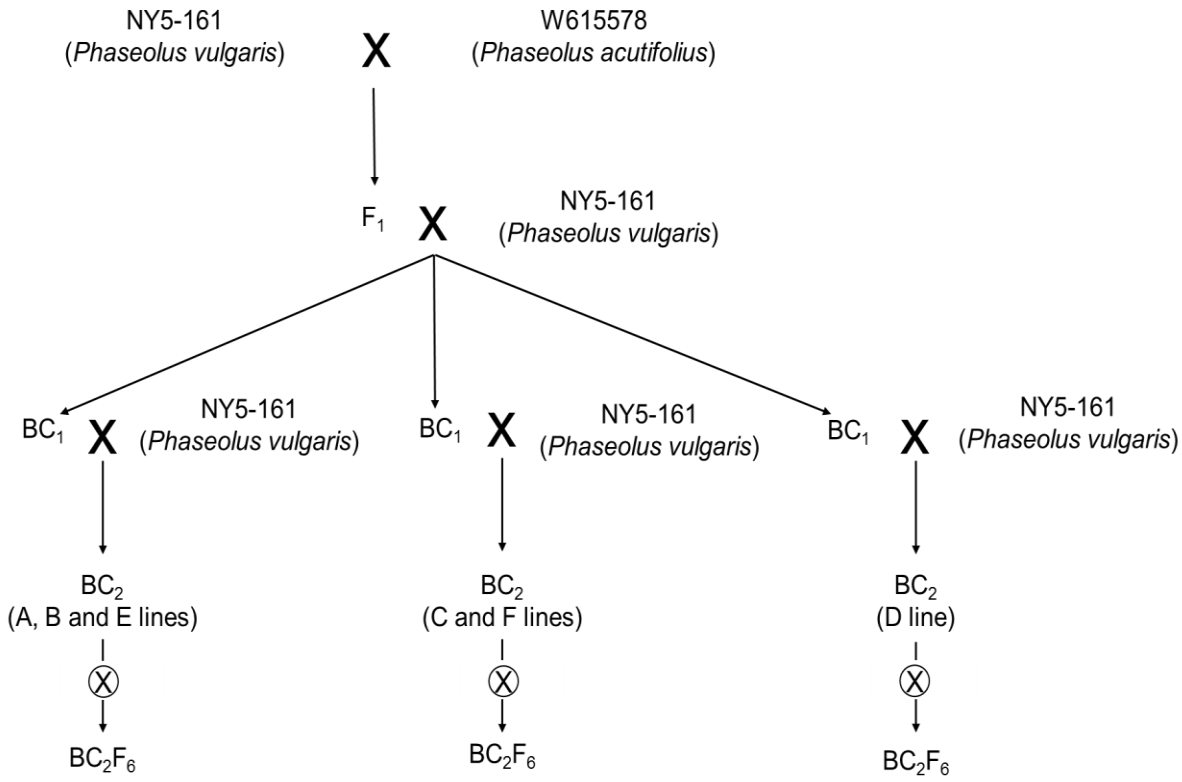


Figure 2.1: Development of the interspecific introgression lines used in the field trials. Modified from Martinez Rojo, 2010.

in the larger germplasm survey, including increases in total ureide, decreases in total ureide or no change. Also, at least one genotype from each independent back cross (Figure 2.1) was selected.

Plants from the first survey were donated (B. Tesfaye) 30 days after planting. As they were being grown for the purpose of DNA extraction, growth conditions were not recorded. In the subset germplasm survey, plants were grown in four inch pots containing Sunshine mix 1 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB) for one month, while kept well-watered. Plants were kept at (24ºC/16ºC day/night temperature) and a 12 hour photoperiod.

Plants were fertilized every 10 days with nutrient solution containing 2 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.312 mM K_2SO_4, 0.5 mM K_2HPO_4 and 25 mM $FeSO_4$/Na_2EDTA, 2.5 mM H_3BO_3, 1 mM $ZnSO_4$., 0.1 mM $NaMoO_4$, 0.11 mM $NiCl_2$, 0.15 mM $CuSO_4$, 0.01 mM $CoCl_2$, 2 mM $MnSO_4$, and 5mM NH_4NO_3.

2.2.2.2. Sampling and treatment

After one month, leaves were sampled using a leaf disk punch (1.77 cm^2). Tepary leaves were too small to exclude the mid-rib, however as leaves were approximately the same size, it was assumed equal amounts of mid-rib was sampled. Two leaf disks were placed into four separate 1.6 mL microfuge tubes: two for treatment and two as a control. Sampling was done two hours into the photoperiod. Three biological replicates were completed.

Leaf disks were submerged in 400 mL of 0.5 mM MV (treatment) or 400 mL of distilled water (control). After 48 hours, the remaining liquid was pipetted out of the tube and the leaf disks were frozen at -80ºC for analysis.

2.2.3. Controlled environment chilling experiment

2.2.3.1. Growth and acclimation conditions and chilling procedure

Tepary bean, common bean and interspecific introgression lines were sown in four inch pots containing Sunshine mix 1 (Sun Gro Horticulture Canada Ltd., Seba Beach, AB) for one month. Plants were fertilized every 10 days with the nutrient solution described above (see 2.2.2.1).

After thirty days, plants were moved to a chamber with 7°C light period and 5°C dark period for potential acclimation for two days.

After the 48 hour acclimation period, leaf disks (1.77 cm^2) were punched and placed into 15 mL conical tubes. The treated tubes were subjected to a cold bath (Haake A82) at 2°C in the dark. Control leaf disks remained at 5°C in the dark. Every half hour the temperature was dropped 1°C, until the cold bath was -2°C, at which it was held for 1 hour. At 0°C ice shavings were added to the tubes to act as an ice nucleator. After 1 hour at -2°C the temperature was increased by 1°C every 20 minutes until the cold bath reached 2°C.

2.2.4. Field trial experiments

2.2.4.1. Water limitation trial

Water limitation trials were conducted in the spring of 2013, 2014 and 2015 at the United States Department of Agriculture - Agriculture Research Service (USDA-ARS) field site in Isabela, Puerto Rico (N18°28.183′ and W67°03.191′; 122m above sea level). The soil is an Oxisol series Cotto clay (approximately 60.4% sand, 28.8% clay and 10.4% lime). Replicates (2 in 2013, 3 in 2014 and 4 in 2015) were planted in a group balanced block in a split plot design with water status as the main plot and genotype as the sub-plot. Seeds (45 per row) were planted in 2.74 m rows, spaced 0.76 m apart, with a border row surrounding the trial. Planting occurred in late January and the trial was irrigated as needed before the treatment period. Irrigation was discontinued to the treatment rows at the initiation of flowering (before four weeks after planting) (see Table 3.2 for dates). Control replicates were watered twice a week after irrigation was discontinued to the treated rows. The field was not inoculated before or during this trial, however as the field was used in previous years, it was assumed that the bean plants were coming into contact with rhizobia and, therefore, were likely fixing nitrogen.

In 2013 and 2014, leaves were collected every two days and dried in a drying oven in Mayaguez, PR at the USDA-ARS Tropical Agriculture Research Station. After the trial was completed, the leaf material was shipped to Canada where it was stored at -20°C until analysis. Ureide content was measured using HPLC (see section 2.3.1) on a dry weight basis. Drought scores

(visual score based on leaf stress symptoms), flowering data, germination percentages and other phenotypic notes were also taken during this period.

Above ground biomass and yield measurements were provided by Dr. Tim Porch (USDA-ARS) and the USDA-ARS field crew after harvest. A 2 m strip was harvested from the center of each plot to avoid edge effects. Dr. Porch and the field crew also provided drought scores in 2014 and 2015.

Climatic data was collected from the United States Department of Agriculture Natural Resources Conservation Service website (www.wcc.nrcs.usda.gov) from data measured at the Isabela Substation. If data were incomplete, measurements from the Mora Camp, PR substation and the Coloso, PR substation were used (and indicated in Appendix D).

2.2.4.2. Sub-zero temperature trial

Sub-zero temperature trials were conducted in three consecutive years, from 2013-2015, in Saskatoon, Saskatchewan (52°07′22.0″N 106°37′22.4″W in 2013 and 52°08′12.4″N 106°37′13.8″W in 2014 and 2015) in the Dark Brown soil zone. Four replicates including 160 accessions of tepary bean, common bean and interspecific introgression lines were planted with a four row seeder (12 inch spacing) in a randomized complete block design on three separate planting dates in August in attempt to subject the population to a sub-zero temperature event at the seedling stage, mimicking the physiological stage that would incur a sub-zero temperature event in a late spring frost. After each sub-zero temperature event, seedlings at approximately the V1 stage (first trifoliate unfolded) were analyzed.

Numerical data collected from the sub-zero temperature trial included number of plants germinated and number of plants alive or dead one day after and seven days after the frost event. In 2013, plants were rescued from the field and grown in a greenhouse to determine if the plants would survive and to collect seed. Temperature was recorded using a Hobo Pro V2 datalogger (Onset, Bourne, MA) (Appendix C-2).

In 2013 and the first planting of 2014 leaf tissue was collected the day before, the day of and the day after the frost, to determine changes to ureide quantity using HPLC (see 3.3.1.1. and 3.3.1.2). Tissue was collected in 1.6 mL microfuge tubes and stored at -80°C. Before analysis,

tissue was transferred to a freeze drier (Labconco Freezone 6; Kansas City, MO.) and tissue was analyzed on a dry weight basis.

2.3. Analysis procedures

2.3.1. Ureide quantification

Frozen plant material was either ground in 10mM tricine buffer (pH 8.75) using an equal amount of chloroform (for the soybean water limitation experiment), a buffer of 75% methanol in water (pH 11.6) (for the small tepary bean survey) or distilled water (for the field trials and the isolated leaf disk experiments). The difference in buffers used was due to the continual fine-tuning of the method during the progression of this thesis. The slurry was vortexed and centrifuged for 25 minutes at 5°C and 18000 rcf. Supernatant was removed and centrifuged again using the same conditions. The resulting supernatant was syringe filtered, using 13 mm syringe filter with 0.2 um PTFE membrane (VWR International Mississauga, ON) into 1.5 mL amber glass HPLC vials (Agilent Technologies, Santa Clara, CA) and analyzed.

High Performance Liquid Chromatography (HPLC) analysis was completed on an Agilent 1200 Series system using a BioRad Aminex HPX-87H Ion Exclusion column (HPLC organic analysis column; 300 mm x 7.8 mm; Bio-Rad; Philadelphia, PA). 10 µL of sample was injected with a needle wash step onto the column in a running phase of 0.00247N H_2SO_4 (filtered using a Zapcap CR 0.2 um nylon filter, Whatman Inc, Pilscataway, NJ). Samples were run for 50 minutes at 0.5 mL/min with a 5 minutes stop time at the end of each run. Column was held at 20.0°C. Samples were analyzed at a wavelength of 191 nm with a bandwidth of 2 nm. Agilent ChemStation for LC 3D systems (Agilent Technologies, Santa Clara, CA) software was used to quantify ureides. Allantoin, uric acid, and allantoate were detected at approximately 19, 21, and 24 minutes, respectively. Elution times were confirmed using standards before every experiment.

2.3.2. Protein assay

Total protein was assayed using a Bicinchoninic Acid (BCA) Protein assay kit (Novagen, Darmstadt, Germany) following manufacturers guidelines. Samples were analyzed in duplicate

and bovine serum albumin (BSA) was used as a standard. Absorbance was measured at 565 nm on a Beckman DU 7400 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada).

2.3.3. Allantoinase assay

The allantoinase assay was based on an analysis of glyoxylate derivatives by Vogels and Van der Drift (1969; 1970). Modifications were made to the methods that were described in Duran & Todd (2012). Leaf tissue (6 leaf disks for the soybean experiments and 4 leaf disks for the bean experiments) was ground in buffer containing 50 mM tricine and 2 mM $MnSO_4$ and was diluted ten-fold in 50 mM tricine, 2 mM $MnSO_4$, 35 mM β-mercaptoethanol. The sample was divided in two: half was used to measure endogenous allantoin and the other half was used for the assay. 300 µL of the sample was kept on ice for a basal measurement of glyoxylate (the end metabolite measured). Another 300 µL of the sample was added to 300 µL of 2 mM allantoin in the same buffer and incubated at 30°C for 30 minutes. After incubation, 250 µL of controls and samples were added to 250 µL 0.15 N HCL and 250 µL 0.33% (w/v) phenylhydrazine and 1.25 mL of water, in a glass test tube in duplicate. Samples were boiled for 2 minutes, and then allowed to cool in an ice bath for 10 minutes. 1 mL of concentrated HCL and 250 µL 1.67% (w/v) potassium ferricyanide were added and samples were incubated for another 10 minutes. Absorbance was measured at 520 nm on a Beckman DU 7400 spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). Standard curves of glyoxylate in concentrations of 0umol/ml to 3umol/ml was used for quantification. The minimum R^2 of the standard curves developed was 0.98.

2.3.4. Reactive oxygen species assays

2.3.4.1. Superoxide

Superoxide was quantified using a procedure modified from Ramel et al (2009). Leaf disks were removed from treatment solutions and patted dry. Tissue was immersed in 1 mL of a 10 mM potassium phosphate solution containing 3.5 mg/ml nitroblue tetrazolium (NBT) and 10 mM sodium azide (NaN_3). Tubes were placed under vacuum in the dark for 90 minutes. Leaves were removed from the solution, patted dry, and ground in 1mL of a 2 M KOH-DMSO (1:1.16) (v/v) solution. Extract was centrifuged at 18000 rcf for 10 minutes. The supernatant was diluted 10-fold

in 2 M KOH-DMSO and absorbance was measured at 630 nm (Ramel et al., 2009). A standard curve was generated with 1-20% NBT solution diluted in 2 M KOH-DMSO with an R^2 of at least 0.90.

Treatment solutions (supernatant) were also measured for changes in superoxide content. Supernatant was added 1:1 (v/v) to the 3.5 mg/mL NBT solution and kept in the dark for 90 minutes. 100 uL of the sample was diluted in 900 uL 2M KOH-DMSO. Further dilutions were carried out if necessary. Readings were corrected for control, treatment and pre-treatment solutions that did not contain leaf tissue.

2.3.4.2. Hydrogen peroxide

A procedure for quantifying H_2O_2 was developed using modifications to procedures used in Ramel et al (2009). Leaf disks were patted with paper towel to remove treatment solutions, then were submerged in 1mL of 1.25 mg/mL of 3,3'diaminobenzidine (DAB) in water (pH 3.8, adjusted with KOH). The tissue was vacuum infiltrated in this solution for 90 minutes. Tissue was patted dry, and ground in 1 mL of 0.2 M perchloric acid ($HClO_4$). Extract was centrifuged at 18000 rcf for 10 minutes. The resulting supernatant was removed and re-centrifuged for another 10 minutes to remove any turbidity. Absorbance at 450 nm was measured. Quantification was completed by means of a standard curve with final H_2O_2 concentration in a range of 2 mM to 80 mM with resulting R^2 of at least 0.93. Standards were diluted in DAB solution. After 90 minutes under vacuum, the solution was diluted 1:1 (v/v) in 0.2 M $HClO_4$.

H_2O_2 content was determined in the supernatant using a similar procedure. The supernatant was added 1:1 (v/v) to the DAB solution and placed in the dark for 90 minutes. The resulting solution was measured at 450 nm.

2.3.5. Electrolyte leakage assay

After the controlled sub-zero temperature or ROS treatments, tissue was moved to 15 mL conical tubes and water was added to a final volume of 8 mL. Using a SympHony SB40C Conductivity Meter (VWR, Radnor, PA, USA), electrical conductivity (EC_F) of the solution was

measured. Solutions that paralleled treatment but did not contain leaf tissue were analyzed as controls (EC_I). Samples were autoclaved for 30 minutes at 121°C to ensure total cell disruption. After solutions returned to room temperature the final electrical conductivity measurement was taken (EC_T). Tubes that did not contain leaf tissue were also autoclaved as a final control (EC_C). Electrolyte leakage was calculated using an equation from Bajji et al. (2001), with modifications (Equation 2.1). Higher percentages indicate greater damage due to treatment.

Equation 2.1:

$$\textit{Electrolyte leakage (\%)} = (EC_F - EC_I) / (EC_T - EC_C) \times 100 \quad [2.1]$$

2.3.6. Lipid peroxidation assay

Lipid peroxidation was measured using an ALDetect™ Lipid Peroxidation Assay Kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) following manufacturer's instructions. Leaf disks, which had been stored at -80°C were ground in liquid nitrogen. Before thawing, 800 mL of ice-cold 20 mM Tris buffer (pH 7.4) with 10 uL of 0.5M butylated hydroxytoluene (BHT) was added to the sample. The BHT addition prevents sample oxidation during homogenization. The slurry was centrifuged at 4°C for 10 minutes at 3000 g. The supernatant was used in the assay as indicated by the kit manual. Lipid peroxidation is quantified by comparison with a standard curve developed with malondialdehyde in the acetal form (TMOP), which is hydrolyzed in the incubation step of the assay (ALDetect™ Lipid Peroxidation Assay Kit User Manual). Absorbance was measured at 586 nm. Amounts for the standard curve ranged from 0 uM to 4 uM. R^2 for all curves was at least 0.98.

2.3.7. RT-PCR procedure

RNA was extracted from four leaf disks after treatment with MV or H_2O_2 using an Omega E.Z.N.A. Plant RNA kit (Omega Bio-tek, Norcross,GA). An RNase-Free DNase I set (Omega Bio-tek) was used to digest any residual genomic DNA. A Quantitect Reverse Transcription kit

(Qiagen, Toronto, Ontario) was used to create cDNA. Primers were either donated, developed from previously published primer sequences, or designed based on CDS sequences accessed from Phytozome 11.0.2 using Vector NTI Advance 11.0 (InforMax; North Bethesda, MD) (Table 2.1). Primers for *CAT* were designed to amplify all identified catalase genes (Gm06g017900, Gm04g017500, Gm14g223500 and Gm17g261700) and primers for UP were designed to amplify all identified ureide permease genes (Gm02g116300, Gm01g07120, and Gm02g12970). PCR cycles included 94ºC denature for 5 minutes, followed by cycles of 94ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 60 seconds followed by 10 minutes at 72ºC, as described by Li et al. 2006. Cycles were repeated 28 times for the amplification of the *SARK*, *ALN*1, *ALN*2, and *CAT* genes and 34 times for the *ALN*3, *ALN*4 and *UP* genes. The PCR reaction was completed using an Eppindorf MasterCycle Gradient thermocycler (Eppindorf, Hamburg, Germany). A fragment of 18S rRNA was used as the quantitative control as it is more stable than reference genes during senescence (Christiansen 2014). Samples were separated on a 1.5% agarose gel run in 1x TRIS-acetate-EDTA buffer and visualized using ethidium bromide. Three independent replicates were examined. Band intensity was quantified using VisionWorksLS software (UVP; Upland, CA).

2.4. Statistical analyses

Unless otherwise indicated, data in this thesis are presented as averages ± standard error. Normality of distribution were confirmed or data were transformed (documented in Appendix A when performed). One- and two-way analysis of variance (ANOVA) were used to assess the effects of factors and the interaction of factors (where appropriate). A Repeated Measures ANOVA was completed for the ureide quantity in the 2013 water limitation trial.

Means were compared at the 95% confidence interval using a post-hoc Tukey test, where not otherwise indicated. In some cases, targeted comparisons were also completed using Student's t-tests. Homogeneity of variance was also determined where stated, using the Levene's test to indicate significance. Correlation analyses used the Pearson Correlation procedure.

The SAS System 9.4 (SAS Institute Inc., Cary, North Carolina, USA) was used for all statistical analysis. PROC GLM was used for all ANOVAs and PROC CORR was used during all correlations.

Table 2.1: Primer sequences

Gene	Primer	Sequence	Source
GmALN1	Forward	CTGTTGTGGAGGGTGTTGCCTTTGCTA	Duran and Todd (2012)
GmALN1	Reverse	GGGGTCACAATGCGTTTGCTG	Duran and Todd (2012)
GmALN2	Forward	TGGTCTCTTAAGTGCTGGTGTTCTTGGTG	Duran and Todd (2012)
GmALN2	Reverse	GATCAAGGTTATCCTCATTAAGCTCCAAA	Duran and Todd (2012)
GmALN3	Forward	GCATCCTCTCGTAAAGTTCCCGCGGAA	Duran and Todd (2012)
GmALN3	Reverse	TGCTTAGACTTCCCCTGCTTACTATATCC	Duran and Todd (2012)
GmALN4	Forward	ACATCCTCTCGTAAAGTTACCGCGGAG	Duran and Todd (2012)
GmALN4	Reverse	TTTCTTGCTTAGACTTCCCCTGCTTAC	Duran and Todd (2012)
GmSARK	Forward	CCGAATSGCTGGGACATTTGGATAT	Li et al. (2006)
GmSARK	Reverse	GATAAAGATCTGCTACAGAGGTAGA	Li et al. (2006)
18SRNA	Forward	CCTTGCTTGTTGCTTTACTAAATAG	Li et al. (2006)
18SRNA	Reverse	ATGCACCTTTTCGTTTGTTTCGGAG	Li et al. (2006)
Catalase	Forward	ATTACTTCCCTTCAAGGTATGATCCTG	Designed from CDS sequence: https://phytozome.jgi.doe.gov
Catalase	Reverse	AYA AAG CAT CAA CCC ATC GGC	Designed from CDS sequence: https://phytozome.jgi.doe.gov
UPS	Forward	TCTTTTCTTCTTGGGGACATGGC	Designed from CDS sequence: https://phytozome.jgi.doe.gov
UPS	Reverse	GGATCTCAGCTTTGTTGATTTTGTC	Designed from CDS sequence: https://phytozome.jgi.doe.gov

3. Results

3.1. Soybean in water-limited conditions

3.1.1. Allantoin and allantoate accumulate in water-limited soybean leaves

Using High Performance Liquid Chromatography (HPLC), allantoin and allantoate were quantified in leaf disks of fully expanded young soybean leaves during conditions of water limitation. Water was withdrawn until soil reached 20% pot capacity (PC) (determined by weight). Soil was kept at 20% PC until day 10, after which irrigation was completely discontinued. To account for potential loss of fresh weight due to water loss, data were examined on both a leaf area basis (from here on referred to as per leaf disk) (Figure 3.1 and 3.2 A,B) and on a fresh weight (FW) basis (Figure 3.1 and 3.2 C,D).

Basal levels of allantoin were approximately 2.6 times higher in nitrogen-fixing soybean leaves than in nitrogen-fertilized soybean leaves (an average of 14.3 nmol/leaf disk and 5.4 nmol/leaf disk, respectively). Determined by two-way ANOVA, under both nitrogen-fertilized and nitrogen-fixing conditions allantoin accumulated in soybean leaves in response to water limitation (Figure 3.1; Table A-1). The increase of allantoin was significant compared to the paired control 6 days after water was removed for leaves from both nitrogen-fertilized and nitrogen-fixing plants, determined by targeted t-tests, and remained significant for the remainder of the analysis. Similar to basal levels, allantoin accumulated to a higher concentration in nitrogen-fixing soybean leaves (110.3 nmol/leaf disk) than nitrogen-fertilized soybean leaves (15 nmol/leaf disk) (Figure 3.1 A,B). When expressed on a FW basis similar results were obtained (Figure 3.1 C,D). The difference between leaves of nitrogen-fixing and nitrogen-fertilized soybean was greater in the stressed leaves than in the control leaves, increasing to over 7 times the allantoin content.

Similar to allantoin, allantoate levels were also higher in nitrogen-fixing controls than nitrogen-fertilized controls (on average 64.7 nmol/leaf disk and 1.3 nmol/leaf disk, respectively), but to a greater extent (approx. 49 times higher). Allantoate also increased in water-limited soybean leaves in response to water deficit (Figure 3.2). Determined by two-way ANOVA, water limitation caused an accumulation of allantoate in the leaves of both nitrogen-fertilized and nitrogen-fixing

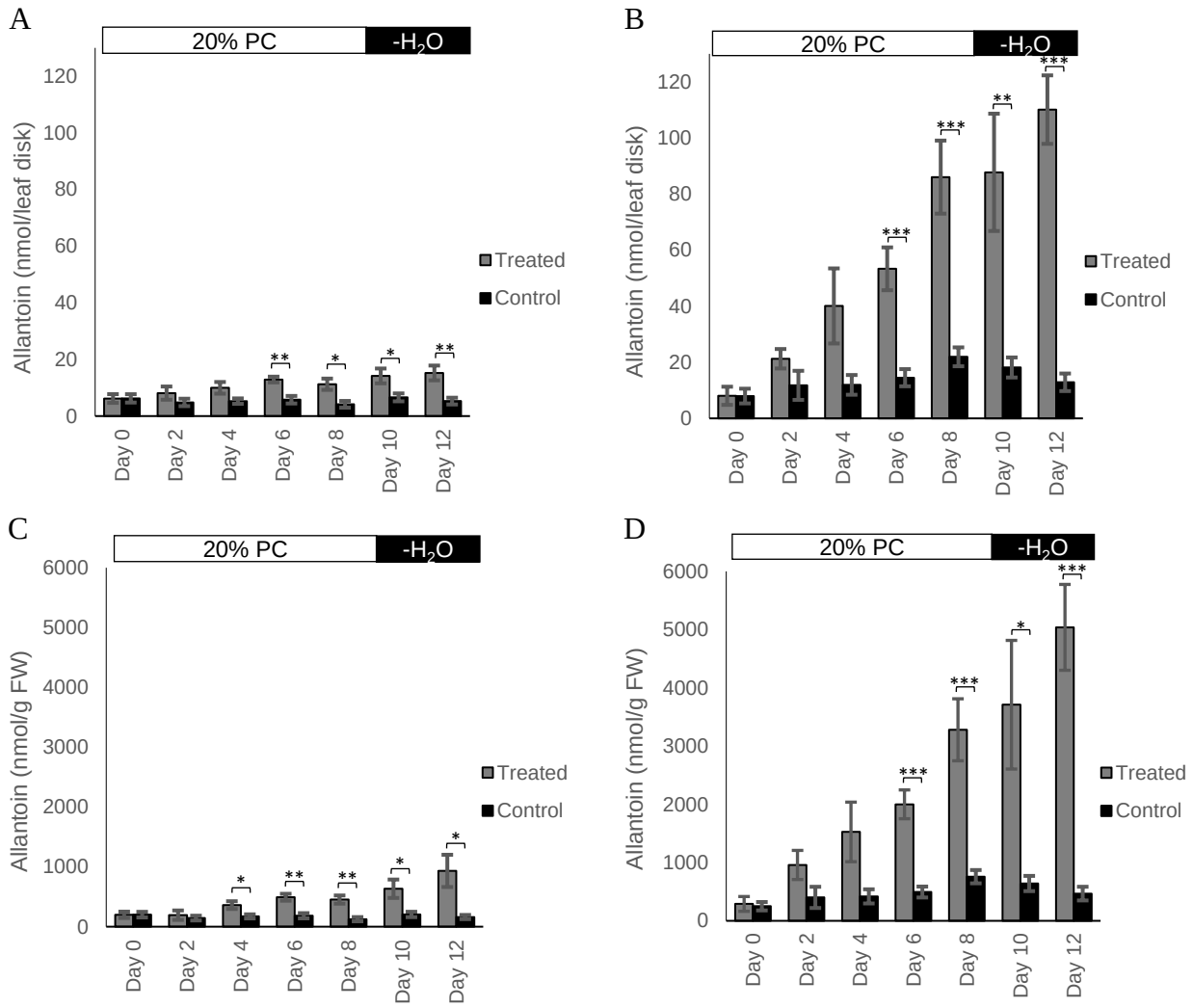


Figure 3.1: Allantoin accumulation in response to water limitation. Allantoin content in fully expanded young leaves of nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) soybean plants. Allantoin was measured as a function of area (leaf disks) (A,B) and fresh weight (FW) (C,D). Data presented are the mean of 5-7 independent replicates ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

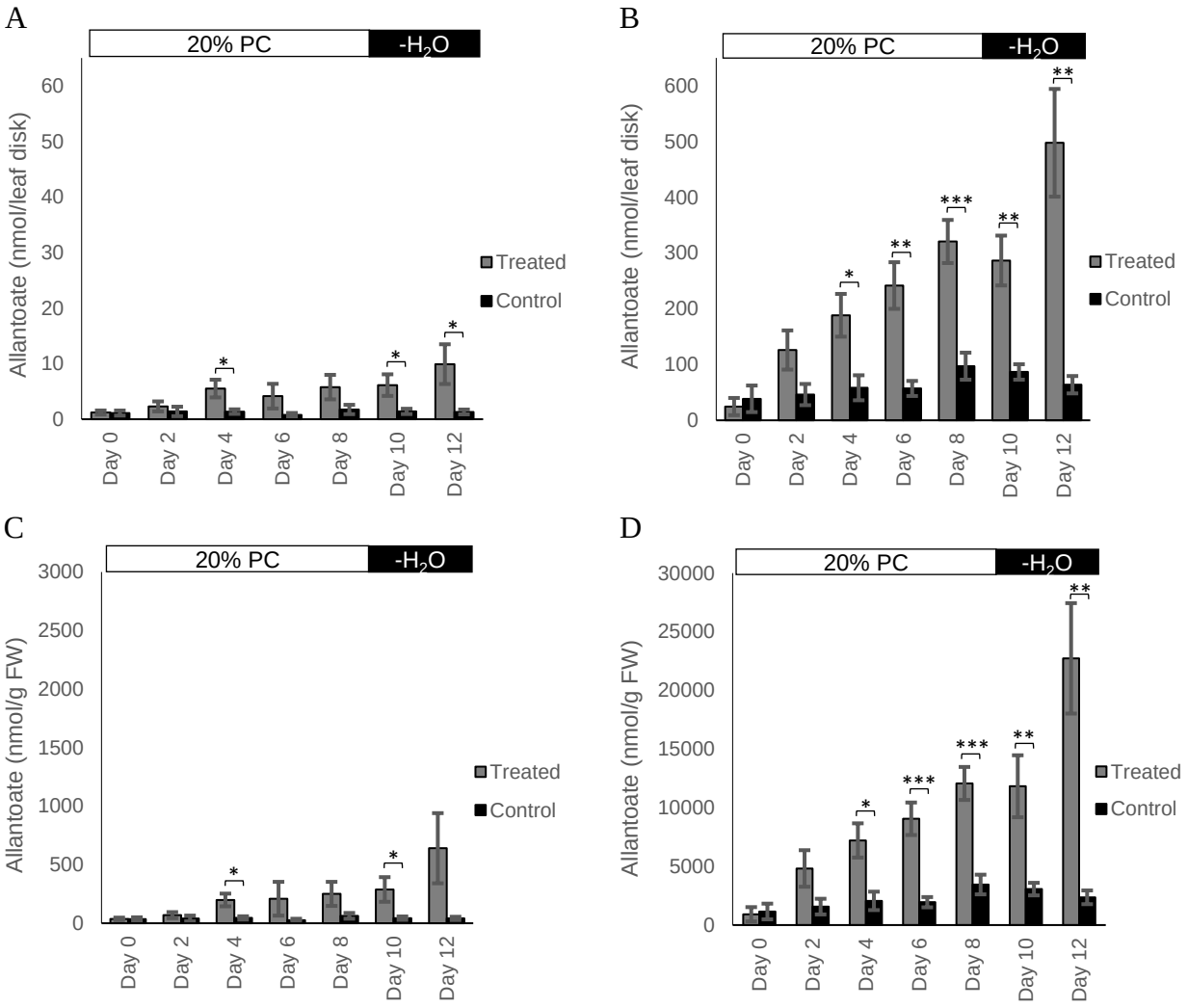


Figure 3.2: **Allantoate accumulation in response to water limitation.** Allantoate content during water limitation in fully expanded young leaves of nitrogen-fertilized (A,C) and nitrogen-fixing soybean plants (B,D). Note the 10x scale differences between the axes for the two nitrogen regimes. Quantity was measured per leaf disks (A,C) and FW (B,D). Data presented are the mean of 5-7 independent replicates ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

plants, when measured per LD and FW (Figure 3.2; Table A-2). In nitrogen-fixing tissue, allantoate accumulated to a significant level on day 4 and remained greater than controls for the rest of the treatment, reaching 497.8 nmol/leaf disk at the end of the analysis period. In nitrogen-fertilized soybean leaf tissue allantoate was greater in treated tissue than in controls on day 4 and again on day 10 and 12. Nitrogen-fertilized soybean accumulated to 9.9 nmol/leaf disk of allantoate in the treated leaves on day 12 (Figure 3.2 A,B). The difference of accumulated allantoate between leaves of nitrogen-fixing and nitrogen-fertilized soybean was approximately 50.2 times higher in the leaves of nitrogen-fixing soybean. Similar results were seen expressing data on a FW basis (Figure 3.2 C,D).

In control nitrogen-fixing soybean tissue, a higher quantity of allantoate was detected than allantoin whereas in control nitrogen-fertilized tissue allantoin was the predominant ureide over allantoate. This relationship was consistent in water-limited tissue, as well (Figures 3.1 and 3.2).

3.1.2. Allantoinase activity in leaves is upregulated during water limitation

Allantoinase assays were performed on leaf tissue of 42 day old nitrogen-fertilized and nitrogen-fixing soybean plants to determine if allantoinase activity varies under water-limited conditions. Six leaf disks from fully expanded young leaves were sampled after available water was limited as described above. Enzyme activity was calculated both per leaf disk (Figure 3.3 A,B) and on a specific activity basis (per mg protein) (Figure 3.3 C,D).

Allantoinase activity increased in leaf tissue during water-limited conditions (Figure 3.3; Table A-3) in leaves of both nitrogen-fertilized and nitrogen-fixing soybean plants. Allantoinase activity per leaf disk in water-limited plants was greater than the control starting on day 8 and for the remainder of the experiment for both nitrogen-fertilized and nitrogen-fixing tissue, determined by targeted t-tests. Specific activity in treated water-limited nitrogen-fixing leaves was only significantly greater than controls on day 8 and day 10, whereas in treated nitrogen-fertilized leaves allantoinase was more active than in controls on day 6 and for every subsequent pair.

Under control conditions, allantoinase activity was greater in nitrogen-fixing soybean leaves (0.087 nkat/leaf disk) than nitrogen-fertilized soybean plants (0.053 nkat/leaf disk). In water-limiting conditions, allantoinase activity was also greater in nitrogen-fixing leaves (0.19

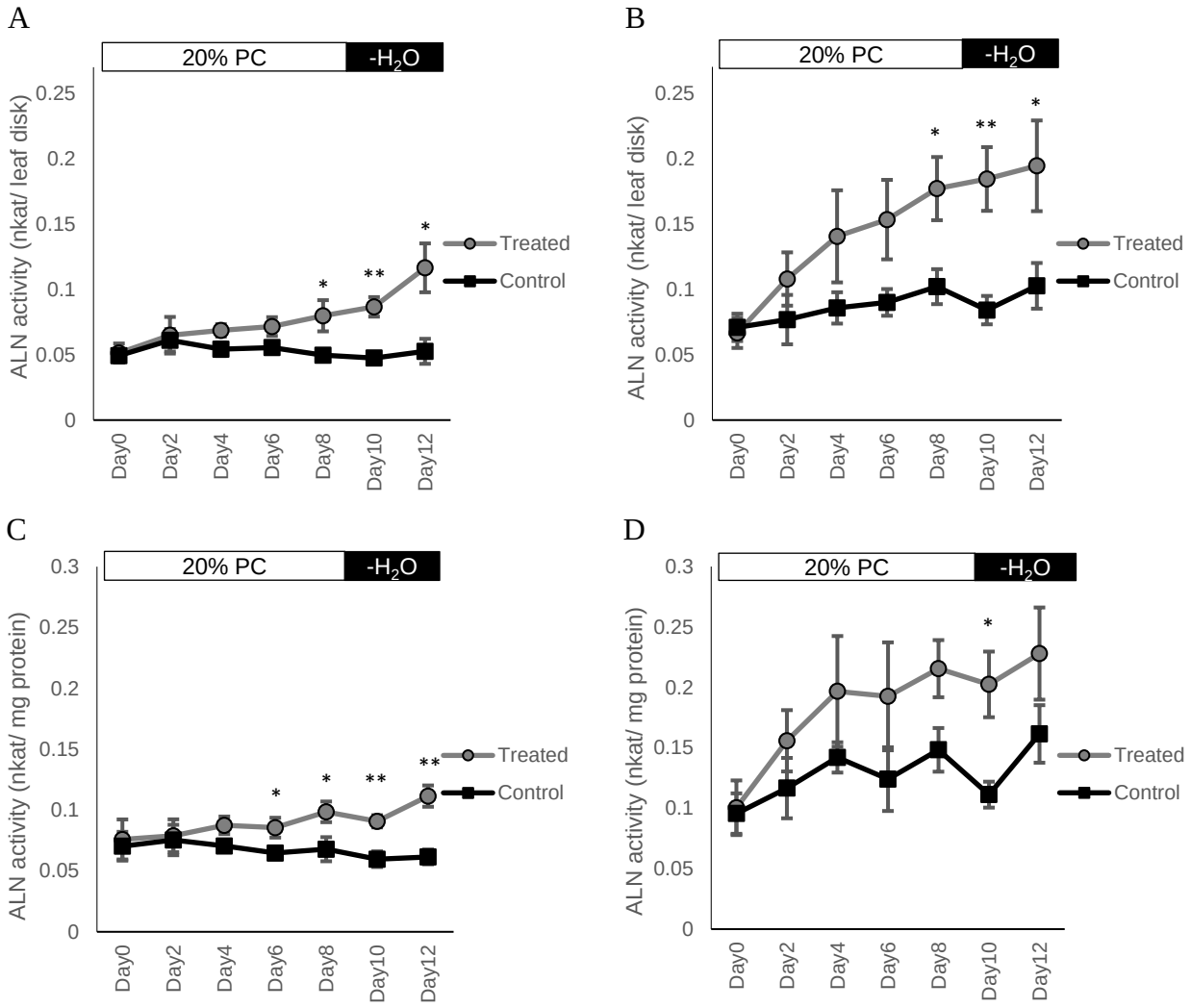


Figure 3.3: Allantoinase activity during water limitation. Allantoinase (ALN) activity in fully expanded young leaves of water-limited (treated) and control soybean plants. Activity was measured in nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) plants. Activity was measured per leaf disk (A,B) and per mg protein (C,D). Data presented are the mean of 5 independent replicates ± standard error. Asterisks indicate significant pairwise differences determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

nkat/leaf disk on day 12) than nitrogen-fertilized soybean leaves (0.12 nkat/leaf disk on day 12) (Figure 3.3).

The change in allantoinase activity in response to water limitation was calculated as the percent of activity in treated leaves compared to activity in control leaves for both nitrogen-fertilized and nitrogen-fixing soybean. A significant change due to time was calculated under both nitrogen sources ($p < 0.001$), which confirmed an upregulation of allantoinase activity under increasing water-limitaiton. No significant difference between the two nitrogen sources or between the interaction of day and nitrogen source was found (Table A-4), indicating that total allantoinase activity was not the same (Figure 3.3), but the relative change in allantoinase activity in response to water limitation was the same under both nitrogen-fertilized and nitrogen-fixing conditions (Figure 3.4). Allantoinase activity increased to approximately twice the basal levels (or 200% of the control) in both leaves of nitrogen-fertilized and nitrogen-fixing soybean under water limitation.

3.2. Effects of induced ROS on ureide metabolism

Soybean leaves were treated with MV to induce endogenous superoxide (O_2^-) production in order to determine whether ureides accumulate in response to increasing ROS levels, which occur during abiotic stress. Ureides were measured by HPLC and allantoinase activity was determined for comparison with metabolic responses that occurred during water limitation.

3.2.1. Allantoin and allantoate increase after treatment with methyl viologen

Allantoin accumulated after treatment with methyl viologen (MV) in soybean leaves, similar to water limitation, however, allantoin concentration was more variable than what was seen in response to water limitation (Figure 3.5 A-D). Determined by two-way ANOVA, treatment with MV caused an increase in allantoin in nitrogen-fertilized soybean leaves, both on an area and a FW basis (Table A-5). Accumulation peaked at approximately 11.5 times higher than control at 48 hours after treatement (Figure 3.5 A,C). Targeted t-tests revealled treated tissue had greater allantoin than control leaf disks at 36 hours and 48 hours post-treatemtent (Figure 3.5 A).

Allantoin also accumulated in leaves of nitrogen-fixing soybean after treatment with MV

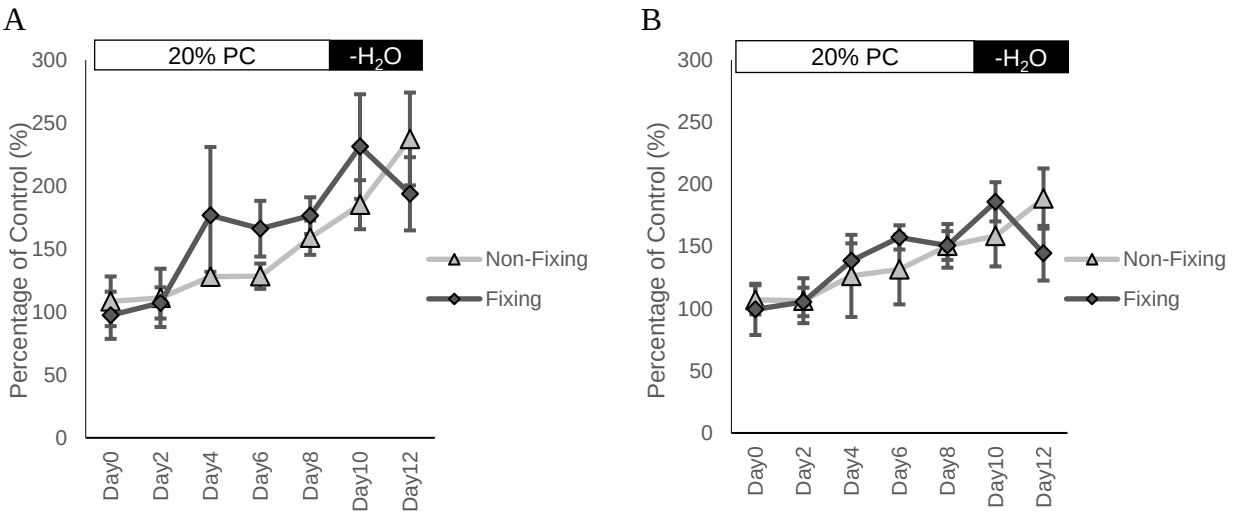


Figure 3.4: Comparison of increase in allantoinase activity between nitrogen-fertilized and nitrogen-fixing soybean. Change in activity between treated and control leaf tissue during water limitation expressed as percent of control. Allantoinase activity was measured in fully expanded young leaves of nitrogen-fertilized and nitrogen-fixing soybean plants. Activity was measured per leaf disk (A) and per mg protein (B). Data presented are means of 5 independent replicates ± standard error. No significant pairwise differences exisited.

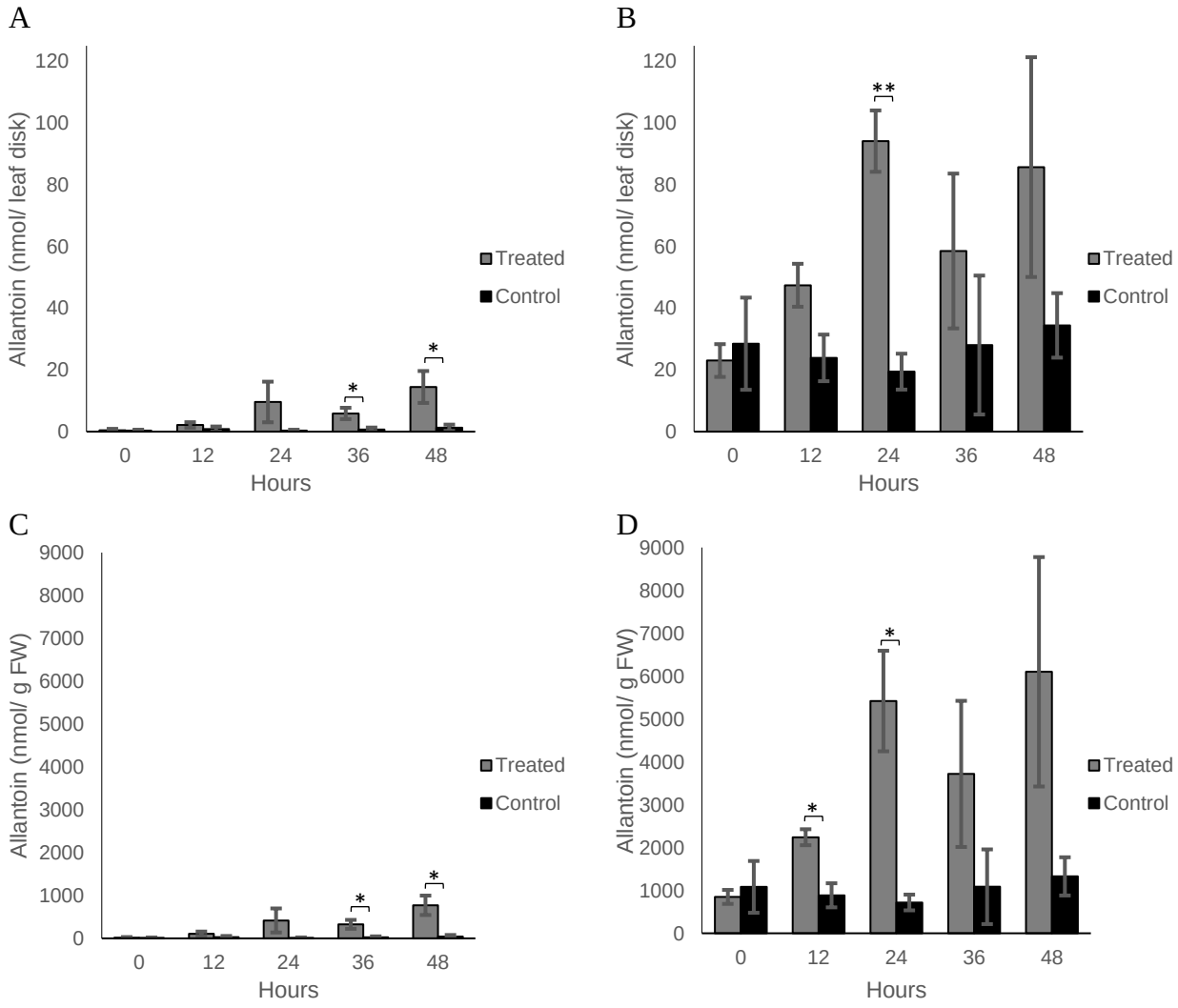


Figure 3.5: Allantoin content in leaves during induced oxidative stress. Allantoin accumulation in fully expanded young leaves after treatment with methyl viologen in nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) soybean plants. Allantoin quantity was measured per leaf disk (A, B) and FW (C,D). Data presented are means of 4 independent replicates for nitrogen-fertilized and 3 independent replicates for nitrogen-fixing ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

(Figure 3.5 B,D; Table A-5). Targeted t-tests revealled significant differences between control and treated tissue at 24 hours on a leaf disk basis (Figure 3.5 B) and at 12 and 24 hours in FW (Figure 3.5 D). The allantoin in the MV-treated leaves increased by 4.7 times the amount in the control leaves. In contrast to leaves of nitrogen-fertilized soybean, in which the accumulation became more pronounced over time, leaves of nitrogen-fixing soybean accumulated allantoin rapidly and demonstrated variability at later stages of analysis (Figure 3.5).

Allantoate accumulated in leaves of nitrogen-fertilized soybean after treatment with MV ($p < 0.05$) (Figure 3.6 A,C; Table A-6). Allantoate also accumulated in leaves of nitrogen-fixing soybean, but was only significant when measured on a FW basis, suggesting the increase in concentration was due to decreased tissue weight (Figure 3.6 B,D). Post-hoc targeted t-tests did not reveal any significant differences between pairs at specific time-points, with the exception of 24 hours after treatment with MV in nitrogen-fixing soybean calculated per FW (Figure 3.6 D). As such, after treatment with MV, allantoin was observed to be the major ureide accumulating due to the stress.

As differences were seen between ureide concentration on a leaf disk and a FW basis, the weight of the leaf disks were compared to determine what changes occurred due to treatment with MV. The FW of the tissue decreased after treatment with MV in leaf disks of both nitrogen-fertilized and nitrogen-fixing soybean (Figure 3.7; Table A-9). MV was used to induce ROS production in the leaves, but given that the leaf FW decreased and the leaves showed visible symptoms of drying, MV likely caused dehydration of the tissue as well.

3.2.2. Allantoinase activity decreases in soybean leaves after MV treatment

Allantoinase activity was assayed to determine the response to treatment with MV. Changes in allantoinase activity during MV treatment differed substantially from water limitation. Activity decreased following treatment with MV instead of increasing as expected (Figure 3.8). The effect of MV was significant when calculated per area in leaves of both nitrogen-fertilized ($p<0.001$) and nitrogen-fixing soybean ($p<0.001$), (Table A-7). Similar results were obtained when data were expressed as specific activity. Treated leaves had lower allantoinase activity than their paired control at 24 and 36 hours after treatment in nitrogen- fertilized soybean (Figure 3.8 A,C)

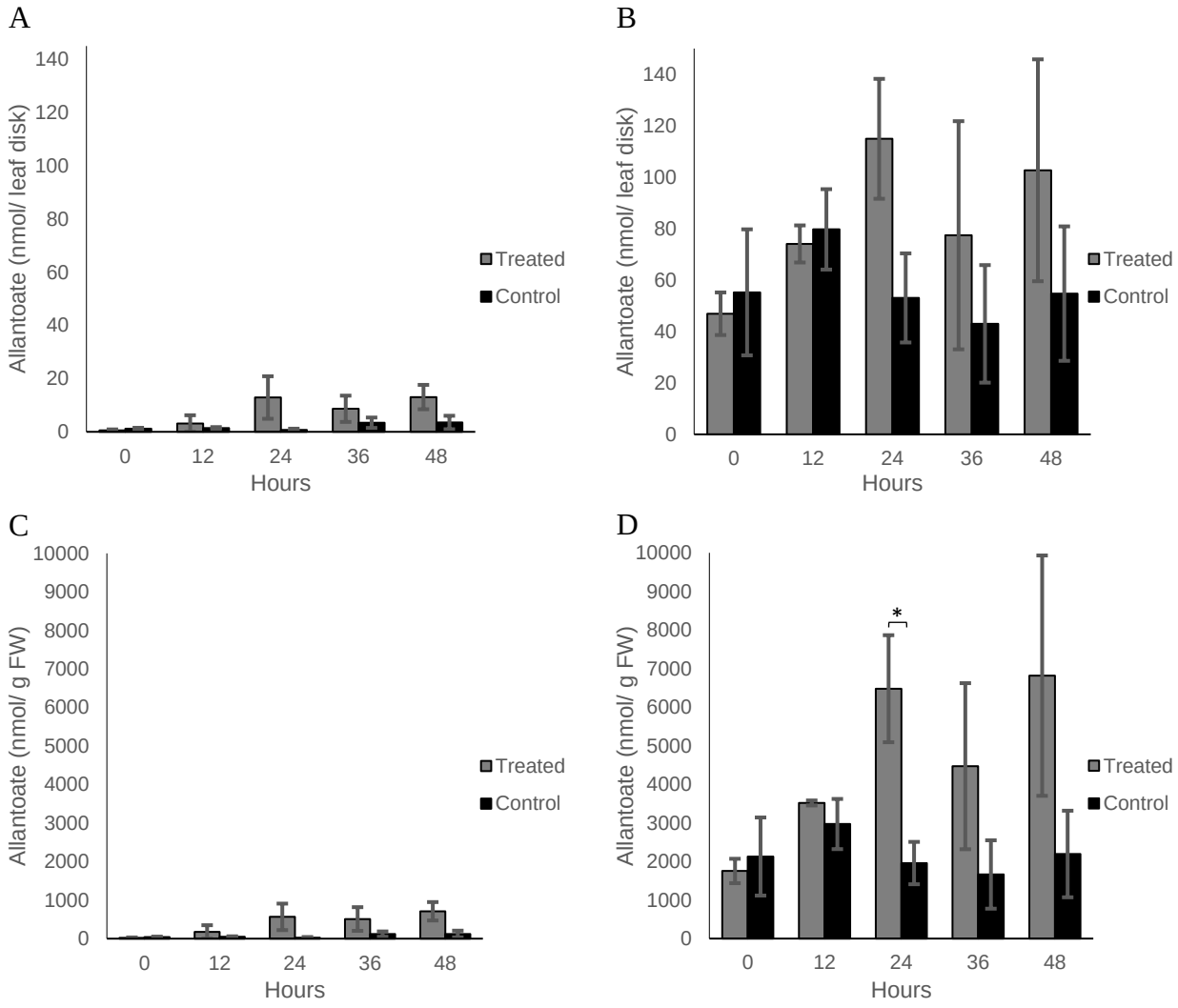


Figure 3.6: Allantoate content in leaves during induced oxidative stress. Allantoate accumulation after treatment with methyl viologen in fully expanded young leaves of nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) soybean plants. Allantoate quantity is depicted as a function of area (leaf disks) (A,B) and FW (C,D). Data presented are means of 4 independent replicates for nitrogen-fertilized and 3 independent replicates for nitrogen-fixing ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

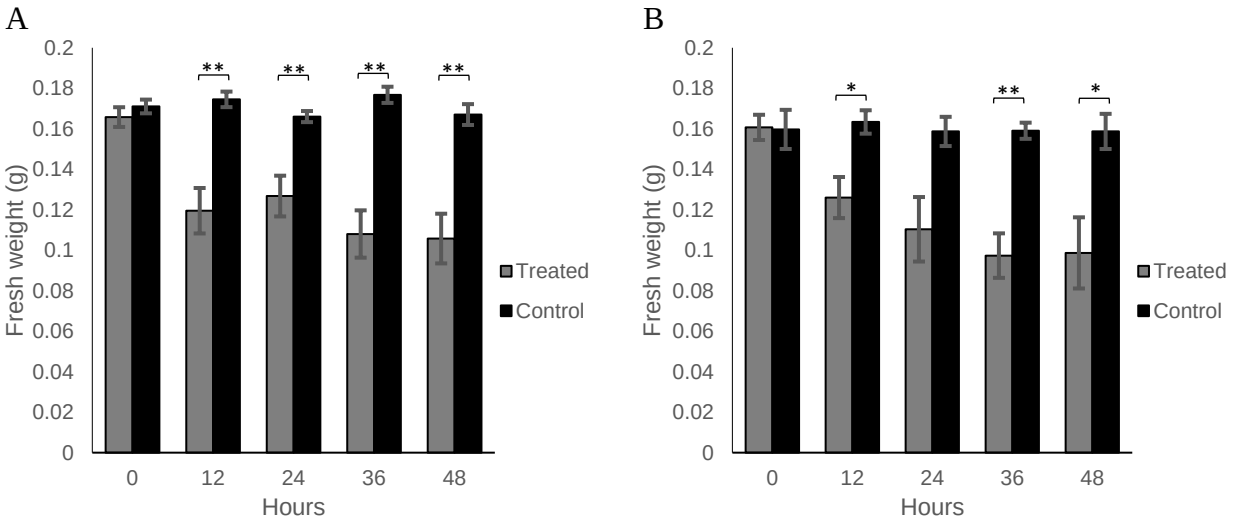


Figure 3.7: Fresh weight of leaf tissue after treatment with MV. Weight of six soybean leaf tissue disks from fully expanded young leaves after treatment with methyl viologen of nitrogen-fertilized (A) and nitrogen-fixing (B) soybean leaf disks. Data presented are means of 4 independent replicates for nitrogen-fertilized and 3 independent replicates for nitrogen-fixing ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

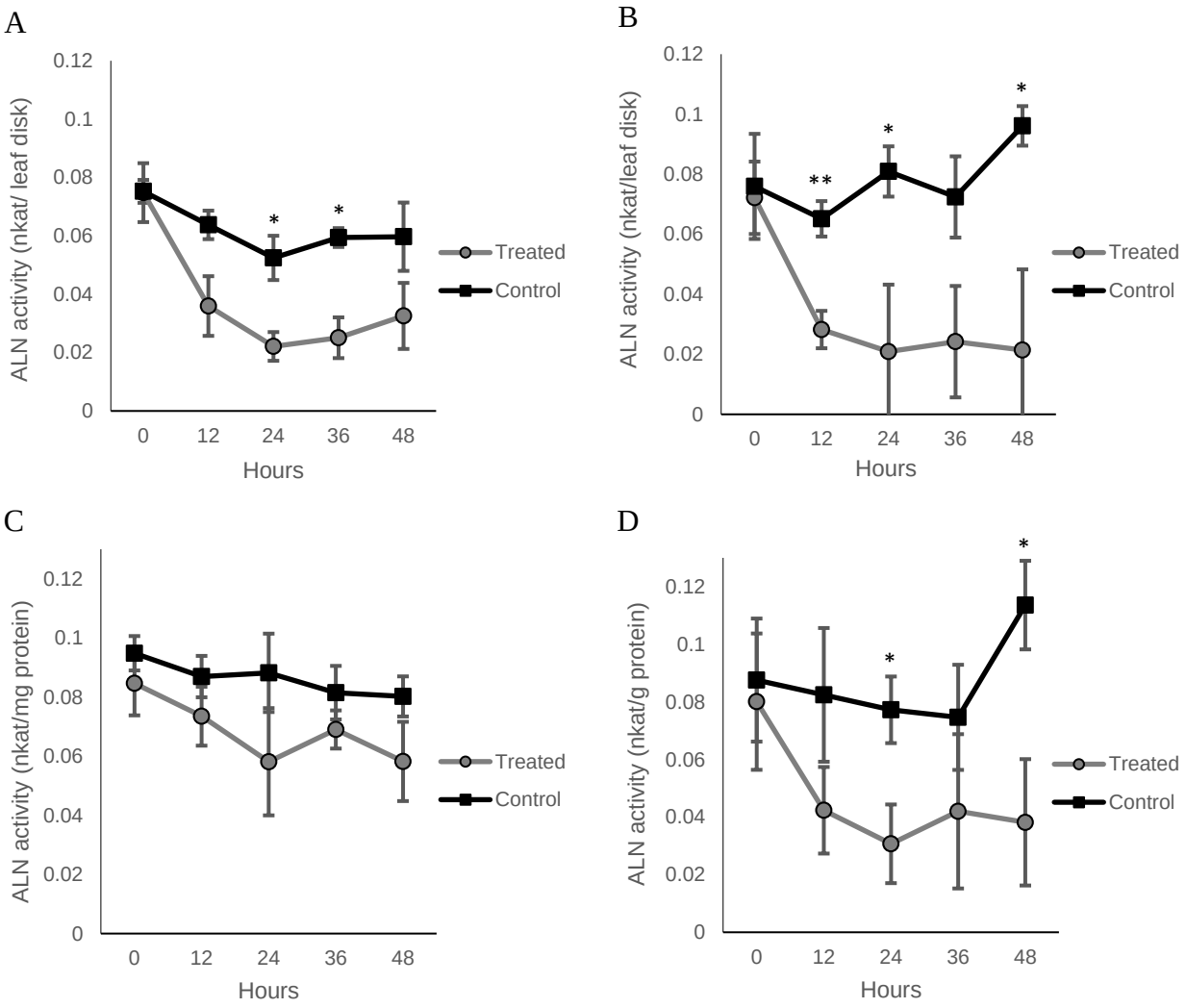


Figure 3.8: Allantoinase activity in leaves during incuced oxidative stress. Allantoinase (ALN) activity in fully expanded young leaves after MV treatment. Activity was measured in nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) soybean plants. Activity was measured per leaf disk (A,B) and mg protein (C,D). Data presented are means of 4 independent replicates for nitrogen-fertilized and 3 independent replicates for nitrogen-fixing ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

and 12, 24 and 48 hours after treatment for nitrogen-fixing soybean (Figure 3.8 B,D) when activity was calculated per LD.

Allantoinase activity after treatment with MV decreased below 45% of a pairwise control for both nitrogen-fertilized and nitrogen-fixing leaf tissue when activity was calculated based on area (LD). Although results were more variable when presented as specific activity, allantoinase activity was still reduced compared to the control (Figure 3.9).

The change in allantoinase activity per leaf disk was not significantly different between nitrogen-fertilized and nitrogen-fixing tissues, however specific activity in nitrogen-fixing tissue decreased to a greater extent when compared to nitrogen-fertilized tissue (determined by a two-way ANOVA, Table A-8). In pairwise comparisons, allantoinase specific activity is only greater in nitrogen-fertilized leaves than nitrogen-fixing leaves 12 hours after treatment (Figure 3.9).

3.2.3. Summary of ureide metabolism response to water limitation and MV

The hypothesis that ureides accumulate during water limitation in both nitrogen-fertilized soybean and nitrogen-fixing soybean was supported. However, although both nitrogen-fixing and nitrogen-fertilized soybean leaves accumulated allantoin and allantoate due to water limitation, the magnitude of accumulation was greater in nitrogen-fixing leaves than nitrogen-fertilized leaves. Allantoinase activity also increased under both nitrogen regimes. The activity was greater in leaves of nitrogen-fixing soybean, however the rate of change was the same in leaves of nitrogen-fertilized soybean and nitrogen-fixing soybean.

The hypothesis that ureide accumulation is due to an increase of ROS was also supported. Treatment with MV, a ROS inducer, primarily caused an accumulation of allantoin, however allantoate also accumulated, especially in the leaves of nitrogen-fixing soybean. In contrast to the water limitation stress experiment, allantoinase activity decreased after treatment with MV. Nitrogen-fixing soybean had a greater relative decrease in leaf allantoinase specific activity compared to leaves from nitrogen-fertilized soybean.

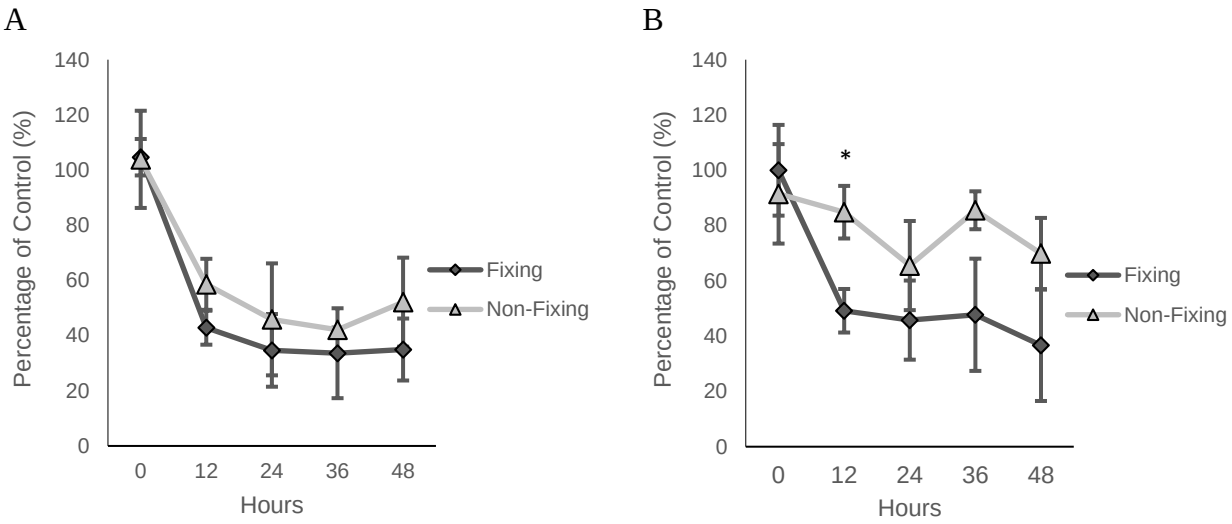


Figure 3.9: Comparison of allantoinase activity between nitrogen-fertilized and nitrogen-fixing plants after treatment with MV. Comparison of change of allantoinase activity in fully expanded leaves of nitrogen-fertilized and nitrogen-fixing soybean plants on an area basis (A) or protein basis (B). Data presented are means of 4 independent replicates for nitrogen-fertilized and 3 independent replicates for nitrogen-fixing ± standard error. Asterisks indicate significant differences between pairs determined by a targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

3.3. Testing the effects of ROS on isolated leaf disks

A preliminary experiment was conducted to determine whether exogenous allantoin or uric acid would reduce the damage from the ROS induced from MV. Leaf disks were taken from nitrogen-fertilized and nitrogen-fixing soybean plants. These disks were floated in a solution containing 0.5 mM MV and either 1 mM allantoin or 1 mM uric acid, as potential scavengers of ROS. Control leaf disks were floated in distilled water. Electrolyte leakage was quantified 24 and 48 hours after treatment.

In comparison to leaf disks that were floated in water, MV-treated leaves exhibited increased electrolyte leakage (Figure 3.10). In expanding this experiment to a full experiment, several changes were made. First, only leaf disks from nitrogen-fertilized leaves were tested, since (i) background ureide levels were likely to be lower, and (ii) they were more responsive to the addition of exogenous allantoin and uric acid in the preliminary experiment (perhaps, in response to (i)). Second, leaf disks in the full experiment were pre-conditioned with allantoin and uric acid for 24 hours, instead of added at the same time as the MV, to allow the leaf tissue time to take up the ureide compounds and reduce any effects of a chemical reaction between the MV and the ureides occurring before uptake by the leaf. Third, hydrogen peroxide (H_2O_2) was added to the experiment as an additional treatment to provide exogenous ROS as a comparison to endogenous ROS generation with MV.

3.4. Effect of ureides on exogenous ROS

An experiment was conducted to determine the effects that ureides have on leaf tissue exposed to exogenous ROS, using H_2O_2. As described above, the leaves would be pre-conditioned for 24 hours and treated for 24 hours (Figure 3.11). During the full 48 hours of the procedure, the leaf disks were kept in the light (approximately an average of 4.7 μmol s^{-1} m^{-2}). Leaves were analyzed for ureide content, ROS content, allantoinase activity, protein content and lipid peroxide content.

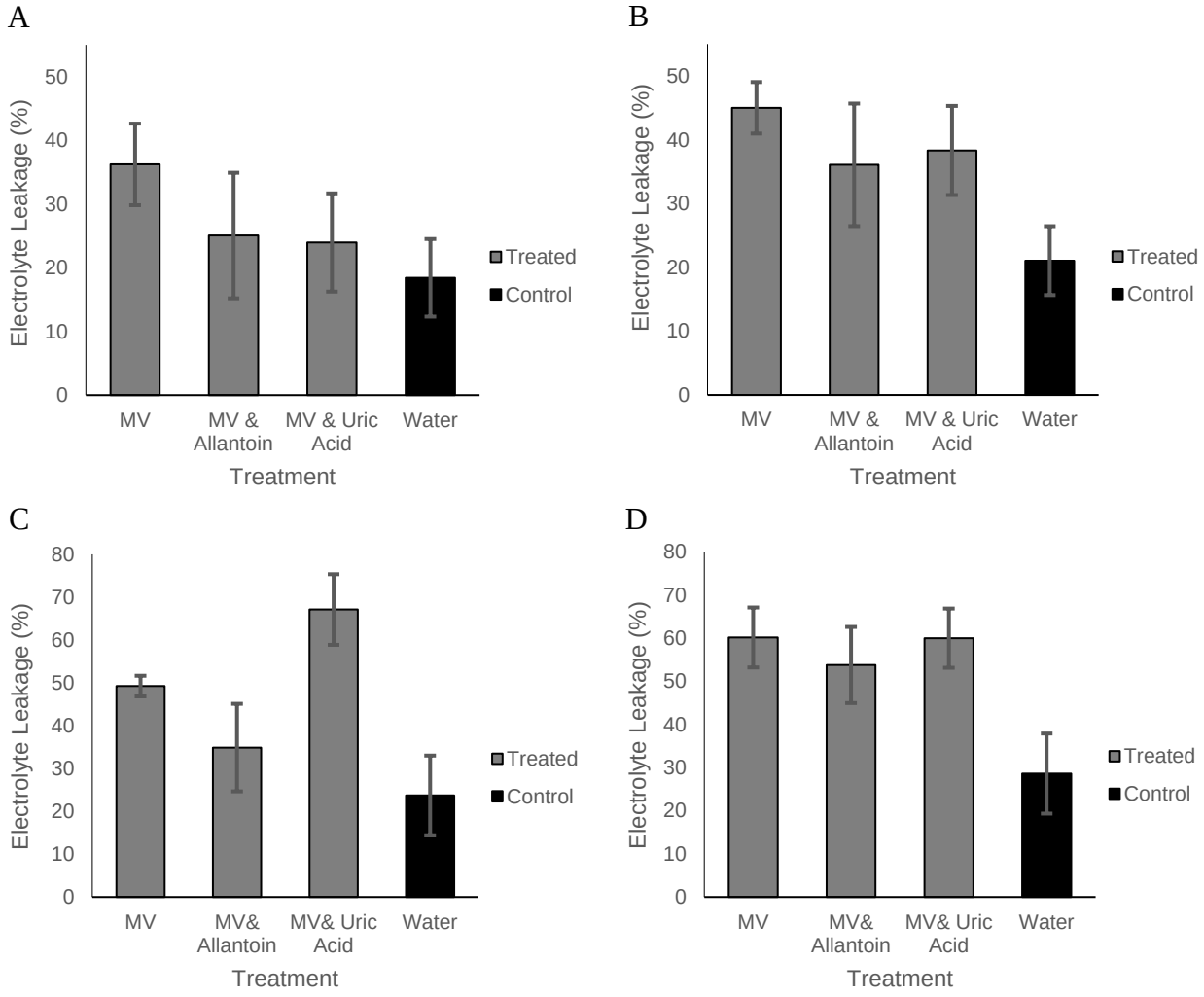


Figure 3.10: Exogenous ureide effect on ROS. A preliminary experiment to determine if exogenous ureide application could have an effect on cell death during an induction of ROS. Nitrogen-fertilized (A,C) and nitrogen-fixing (B,D) soybean leaves were analyzed using an electrolyte leakage assay. Analysis was completed 24 hours (A,B) and 48 hours (C,D) after treatment with water (control), MV, MV and allantoin, and MV and uric acid. Controls were leaf disks floated in distilled water. Data presented are means of 5 independent replicates ± standard error.

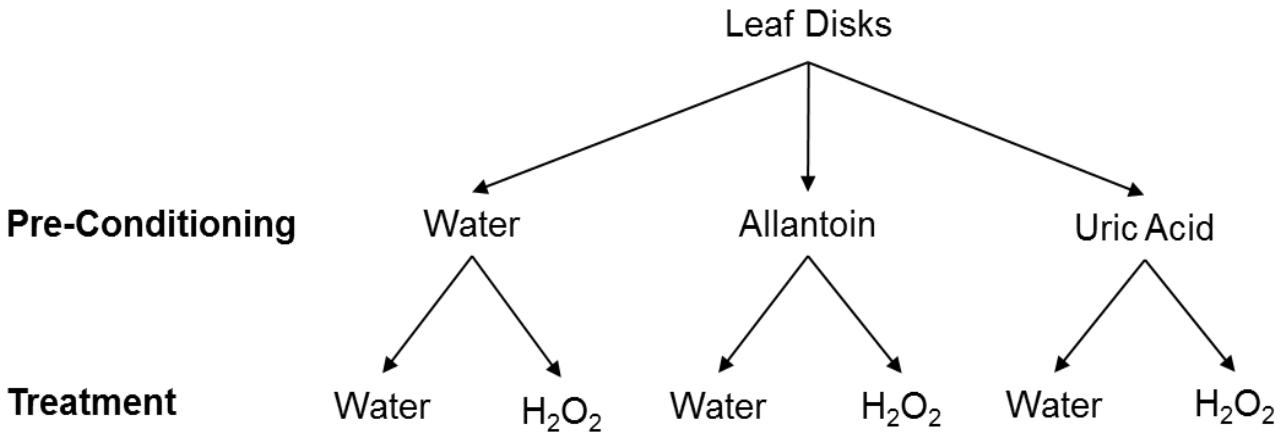


Figure 3.11: A diagram of the exogenous ROS treatment.

3.4.1. Allantoin and uric acid pre-conditioning reduces cell death caused by H_2O_2 treatment

Leaf disks were analyzed by HPLC to determine ureide content after pre-conditioning with allantoin and allantoate and after treatment with H_2O_2. Pre-conditioning with either allantoin or uric acid resulted in uptake of each ureide compound after 24 hours, increasing in concentration after 48 hours in the absence of H_2O_2 (Table B-3). The leaf disks pre-conditioned in water contained allantoin, averaging 9.4 nmol/leaf disk and trace amounts of uric acid. After treatment with H_2O_2, allantoin levels significantly decreased and uric acid levels increased slightly to 0.7 nmol/leaf disk, however, the increase in uric acid was not significant (Figure 3.12). No allantoate was detected in any of these samples.

The allantoin pre-conditioned leaves that were not treated with H_2O_2 contained an average of 40.6 nmol of allantoin per leaf disk and 98.9 nmol of allantoate per leaf disk. A trace amount of uric acid was also detected in some samples. Leaves treated with H_2O_2 had less allantoin (18.8 nmol/leaf disk) and allantoate (23.3 nmol/leaf disk), and contained more uric acid (1.7 nmol/leaf disk) than the control leaves (Figure 3.12). H_2O_2 had a similar effect on leaf disks floated in uric acid, causing decreases in both uric acid and allantoin compared to untreated controls, although not significantly (Figure 3.12).

Water pre-conditioned plants showed the largest average difference in average O_2^- content, increasing to 52.2 mmol NBT/ leaf disk from 43.3 mmol NBT/leaf disk. However, O_2^- content in the leaf disks did not significantly differ due to the pre-conditioning or the treatment, determined by two-way ANOVA (Table A-10). Interestingly, the allantoin pre-conditioned leaves showed virtually no change in superoxide content, however differences between pre-conditioning solutions were not significant (Figure 3.13).

H_2O_2 was greater in treated leaf disks compared to control tissue ($p < 0.001$), but no effect was significant due to the pre-conditioning solutions or between the interaction of pre-conditioning solution and treatment, determined by two-way ANOVA (Table A-12). Targeted t-tests between the paired samples, however, indicated a significant difference between treated and control tissue in the water and allantoin pre-conditioned tissue. In contrast, leaf tissue pre-conditioned with uric acid did not statistically differ between the treatment and the control (Figure 3.13).

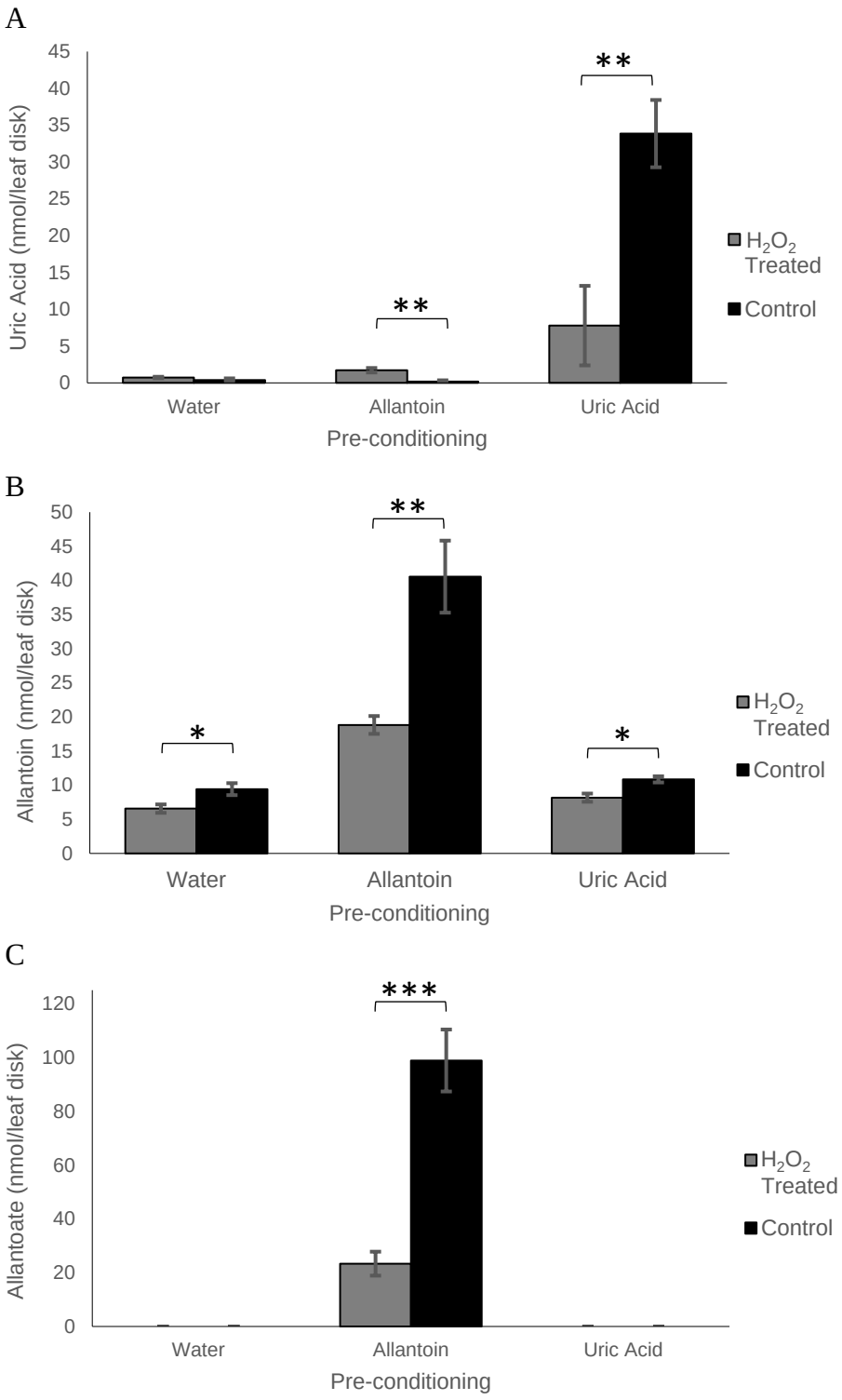


Figure 3.12: Ureide content in leaf tissue after treatment with H_2O_2. Uric acid (A), allantoin (B), and allantoate (C) quantity in nitrogen-fertilized leaves after 24 hours of conditioning in water, allantoin and uric acid followed by 24 hours with exogenous ROS treatment (H_2O_2). Controls were an equivalent amount of distilled water. Data are the mean of 4-6 independent repicates ± standard error. Asterisks indicate significant differences determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

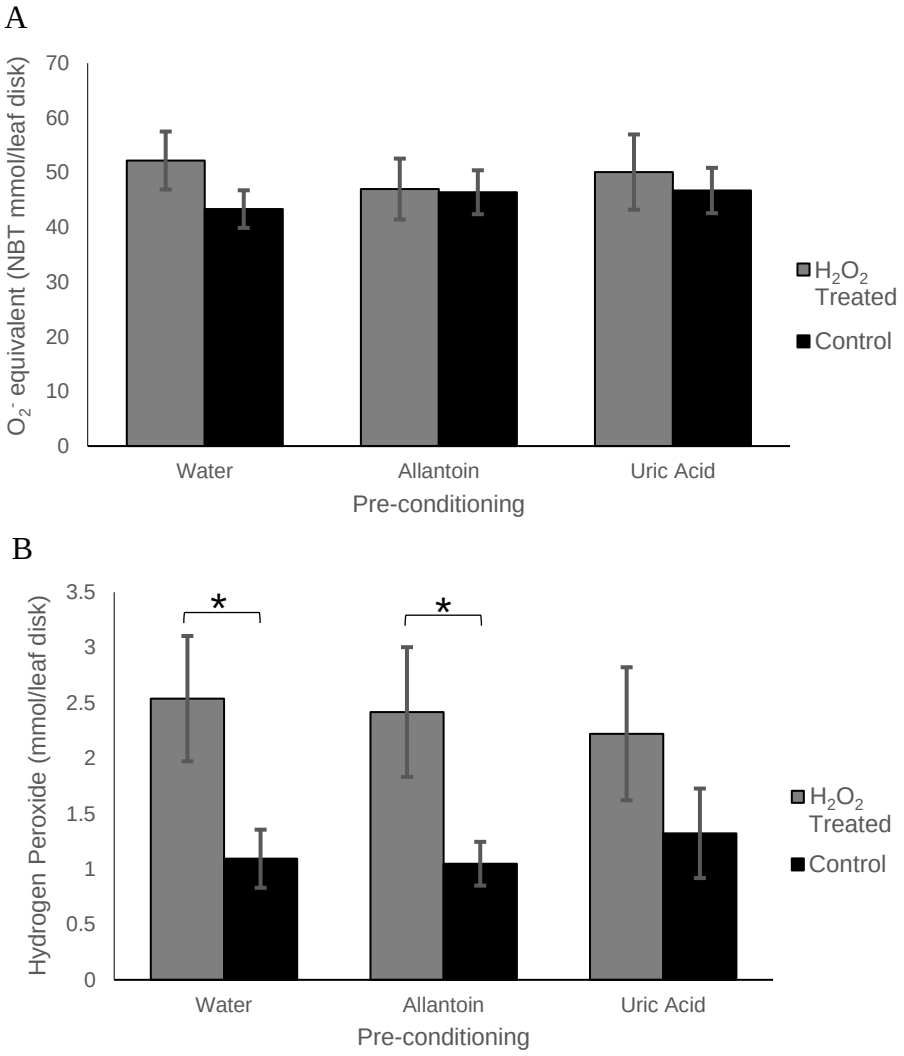


Figure 3.13: ROS content in leaf tissue after treatment with H_2O_2. O_2^- (A) and H_2O_2 (B) content in soybean leaves pre-conditioned with water, allantoin or uric acid and treated with H_2O_2. Data represents averages of 8-9 replicates for O_2^- and 6-7 replicates for H_2O_2 ± standard error. Asterisks indicate significant differences determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

Electrolyte leakage was measured to determine the degree of membrane damage and cell death. Exogenous H_2O_2 induced cell death (Figure 3.14). Tissue from the different pre-conditioning solutions reacted differently to the stress (Table A-14). In the control tissue, cell death ranged from 11.6% in water pre-conditioned tissue to 14.9% in allantoin pre-conditioned tissue. However, using a targeted one-way ANOVA, no difference was significant between the control tissues. After treatment with exogenous ROS, differences occurred between the pre-conditioned tissues. Both the allantoin pre-conditioned leaf disks (25.8% cell death) and the uric acid pre-conditioned leaf disks (24.6% cell death) had less electrolyte leakage than the leaf disks pre-conditioned in water (32.6% cell death) (Figure 3.14).

3.4.2. Allantoinase activity and protein content decrease after H_2O_2 treatment and lipid peroxides increase

Allantoinase activity was measured to determine changes in ureide metabolism after treatment with exogenous ROS. In the control tissue, average allantoinase activity was lower in the water pre-conditioned leaves (0.078 nkat/mg protein) and in the uric acid pre-conditioned leaves (0.080 nkat/mg protein) than in the allantoin pre-conditioned leaves (0.092 nkat/mg protein) although no relationships were statistically different. The same trend was seen in the treated leaf tissue. Allantoin pre-conditioned leaves had more allantoinase activity (0.68 nkat/mg protein) than the water-preconditioned tissue (0.054 nkat/mg protein). Uric acid pre-conditioned tissue also had a lower level of allantoinase activity (0.57 nkat/mg protein) but it was not significantly different than the allantoin pre-conditioned tissue (Figure 3.15). Specific activity was reduced due to treatment and the pre-conditioning solution had an effect on allantoinase activity, determined by two-way ANOVA (Table A-16).

In the control tissue, the amount of protein ranged from 10.6 mg to 11 mg. In the treated tissue, protein levels ranged from 6.8 mg in tissue pre-conditioned with allantoin to 7.7 mg in the tissue pre-conditioned with uric acid (Figure 3.16). H_2O_2 treatment decreased the amount of soluble protein in soybean leaf tissue, but the decrease was not significantly different between pre-conditioning solutions, determined by two-way ANOVA (Table A-17).

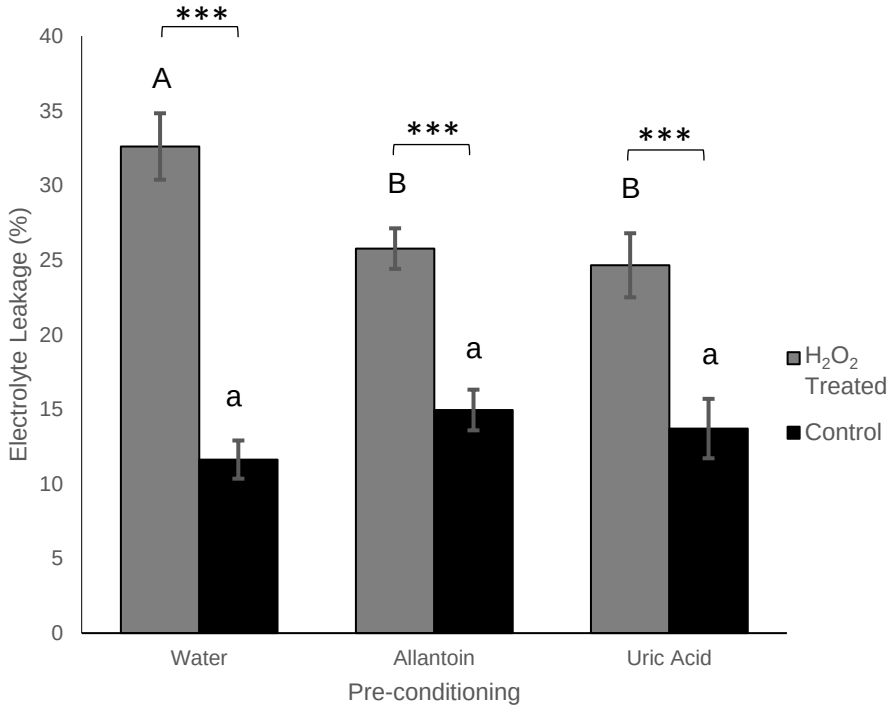


Figure 3.14: Cell death after treatment with H_2O_2. Electrolyte leakage was used to determine cell death in leaf disks after pre-conditioning with water, allantoin and uric acid then treatment with H_2O_2. Data presented are averages of 20 independent replicates for controls and 13-14 independent replicates for H_2O_2 treated ± standard error. Columns with different upper case or lower case letters exhibit significant differences between pre-conditioning solutions ($p<0.05$). Asterisks indicate difference due to treatment with hydrogen peroxide determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

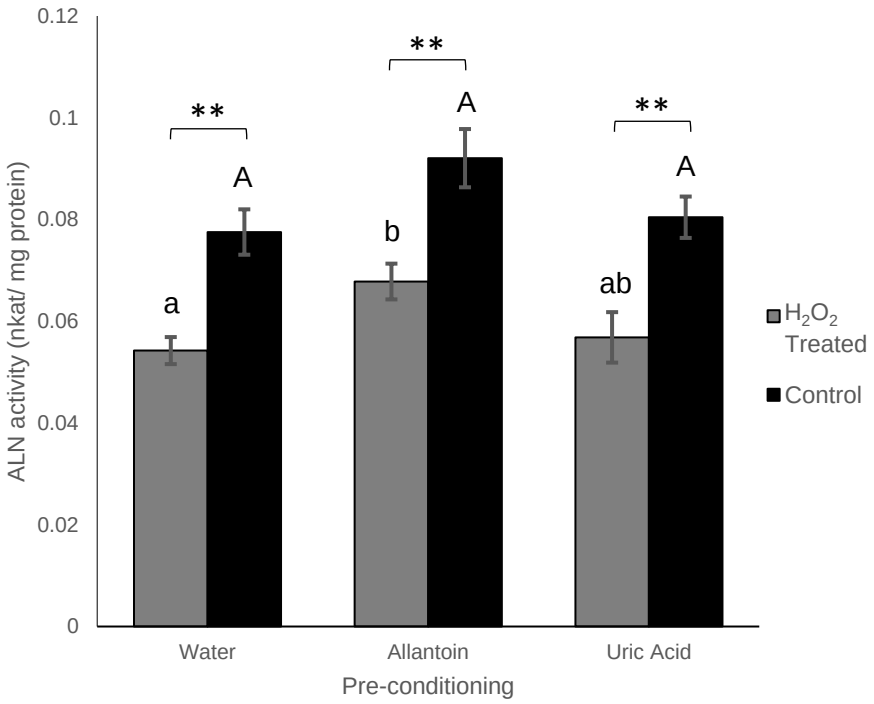


Figure 3.15: Allantoinase specific activity after treatment with H_2O_2. Allantoinase activity in soybean leaf disks pre-conditioned with water, allantoin or uric acid then treated with H_2O_2. Data presented are averages of 7 independent replicates for control and 5 independent replicates for treatment ± standard error. Columns with different upper case or lower case letters exhibit significant differences between pre-conditioning solutions ($p<0.05$), determined using a Tukey's test. Asterisks indicate difference due to treatment with hydrogen peroxide determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

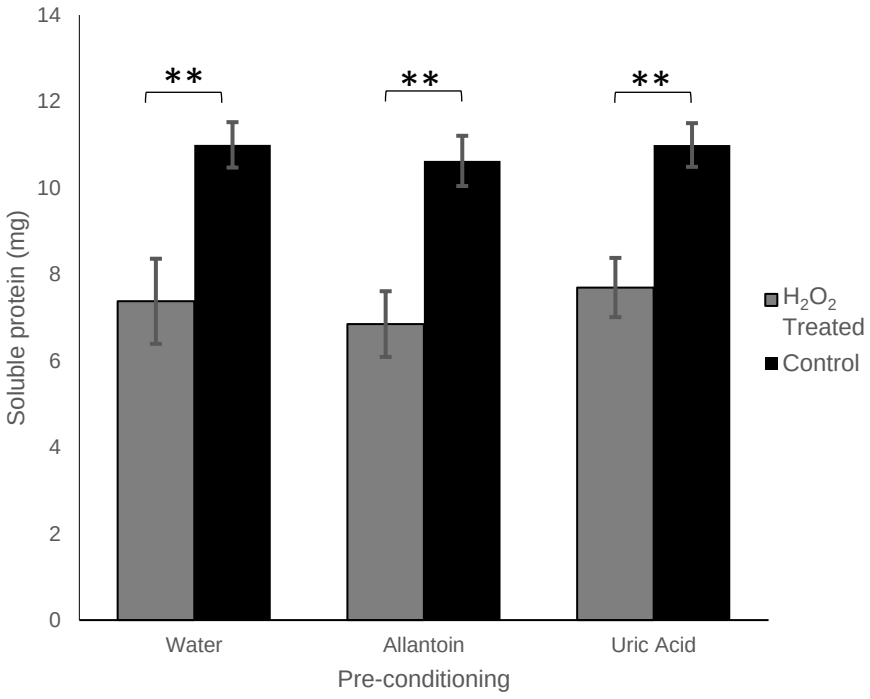


Figure 3.16: Protein content after treatment with H_2O_2. Soluble protein content in leaf disks from fully expanded young soybean leaves. Disks were pre-conditioned in water, allantoin or uric acid and treated with H_2O_2. Data presented are averages of 7 independent replicates for controls and 6 independent replicates for treatment ± standard error. Asterisks indicate difference due to treatment with hydrogen peroxide determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

Lipid peroxides were measured to determine if pre-conditioning with allantoin or uric acid would reduce the amount lipid damage caused by H_2O_2. The allantoin pre-conditioned control leaves had the lowest average level of lipid peroxides (0.25 umol MDA/g protein) compared to the water and uric acid pre-conditioned controls (0.29 umol MDA/g protein for each) (Figure 3.17). Average lipid peroxides were greater in the all pre-conditioned leaves after treatment with H_2O_2. The increase in lipid peroxides after the H_2O_2 treatment was significant, determined by two-way ANOVA ($p < 0.05$) (Table A-18). No pair-wise differences were significant between treated and control tissue.

3.4.3. Transcriptional changes after H_2O_2 treatment

Gene expression was examined after treatment with H_2O_2. Genes tested included *SARK* (senescence associated receptor-like kinase), *ALN1*, *ALN2*, *ALN3* and *ALN4* (allantoinase), *UP* (ureide permease), and *CAT* (catalase). Degenerate primers were designed for *UP* and *CAT* to amplify all genes encoding the ureide permease (three possible genes) and catalase enzymes (four genes) in soybean (Table 2.1). A fragment of 18S rRNA was used as a reference control because it has been demonstrated to be more stable than other common reference genes during tissue senescence (Christiansen 2014). Visually, it appeared as if *UP*, *ALN1*, *ALN2* and *CAT* had decreased expression after treatment. *ALN3* may have increased in expression and *ALN4* and *SARK* expressions did not change (Figure 3.18). Differences between pre-conditioning solutions were not immediately obvious.

When images from RT-PCR agarose gels were quantified, *ALN1* decreased in expression, although was only significant in tissue pre-conditioned with allantoin. Expression of *ALN2* decreased in tissue pre-conditioned with any of the solutions (Figure 3.19 A). None of *ALN3*, *ALN4*, *SARK*, or *UP* had a change in expression. *UP* expression decreased under all replicates, but the variability between replicates was large and may have masked any trend. Uric acid

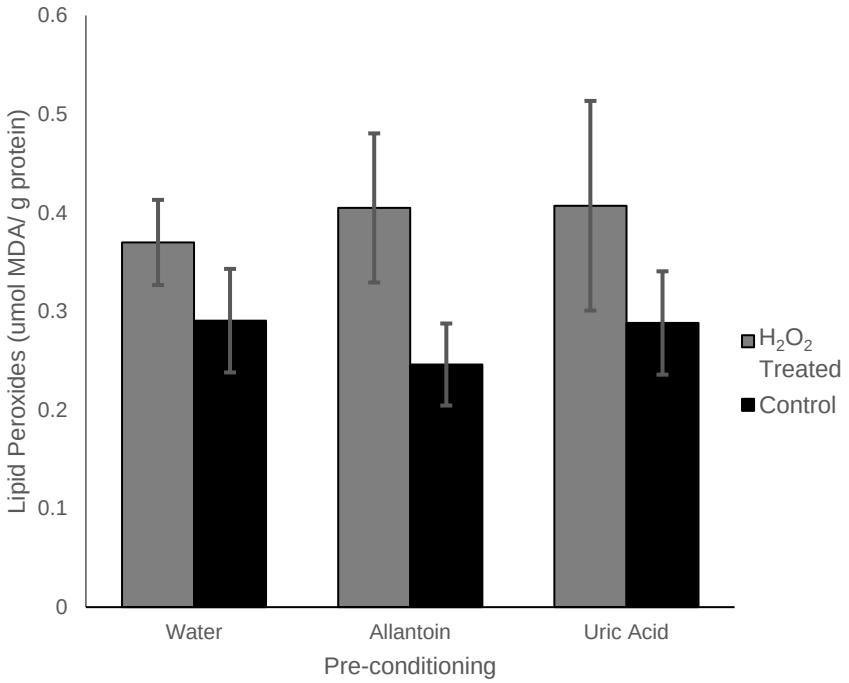


Figure 3.17: Lipid peroxide after treatment with H_2O_2. Lipid peroxides per mg of protein in soybean leaf tissue pre-conditioned with water, allantoin, or uric acid and treated with H_2O_2. Data shown is the average of four replicates ± standard error. Asterisks indicate significance between pairs (p-value: <0.05*, <0.01**, <0.001***).

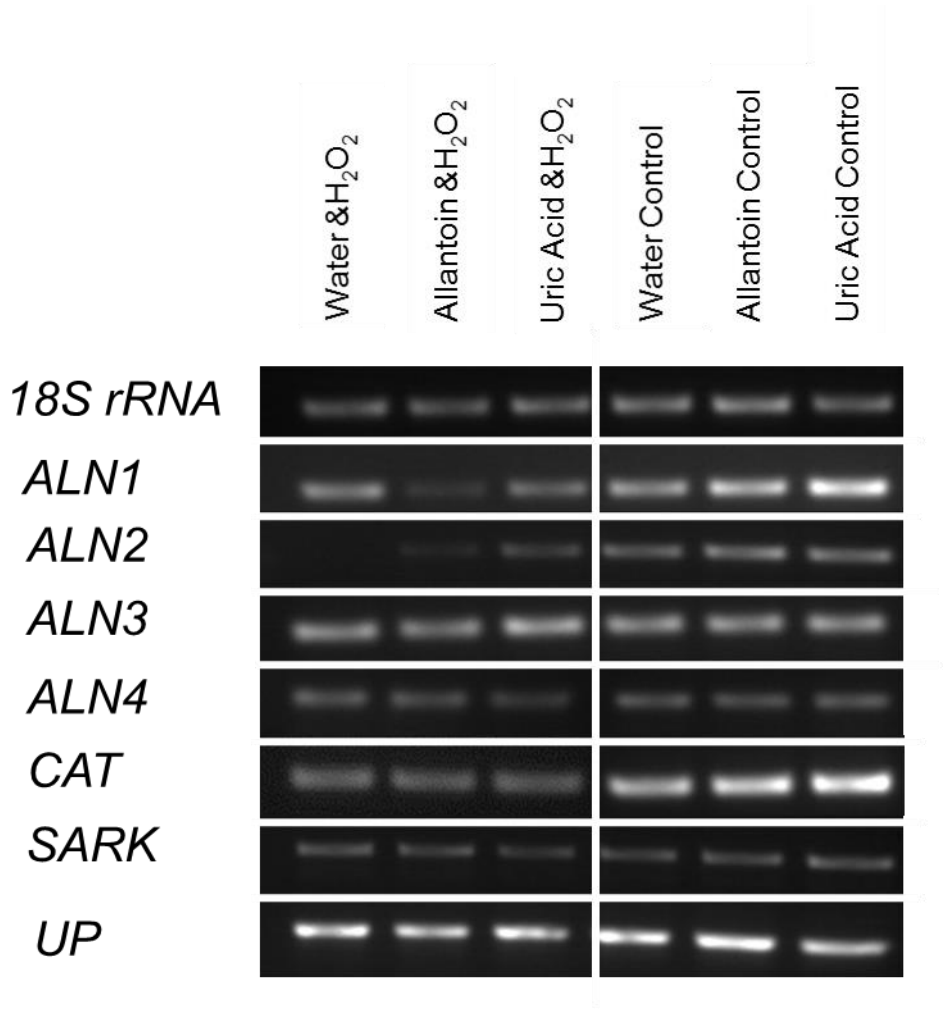


Figure 3.18: Gene expression in soybean leaf tissue after treatment with H_2O_2. Expression of genes in leaf disks of fully expanded young soybean leaves after pre-conditioning with water, allantoin or uric acid and treatment with H_2O_2. *SARK*: senescence associated receptor-like kinase; *ALN*: allantoinase; *CAT*: catalase; *UP*: ureide permease. Reference gene is a 209bp fragment of soybean 18S rRNA. Shown is a representative image from one of three independent replicates completed.

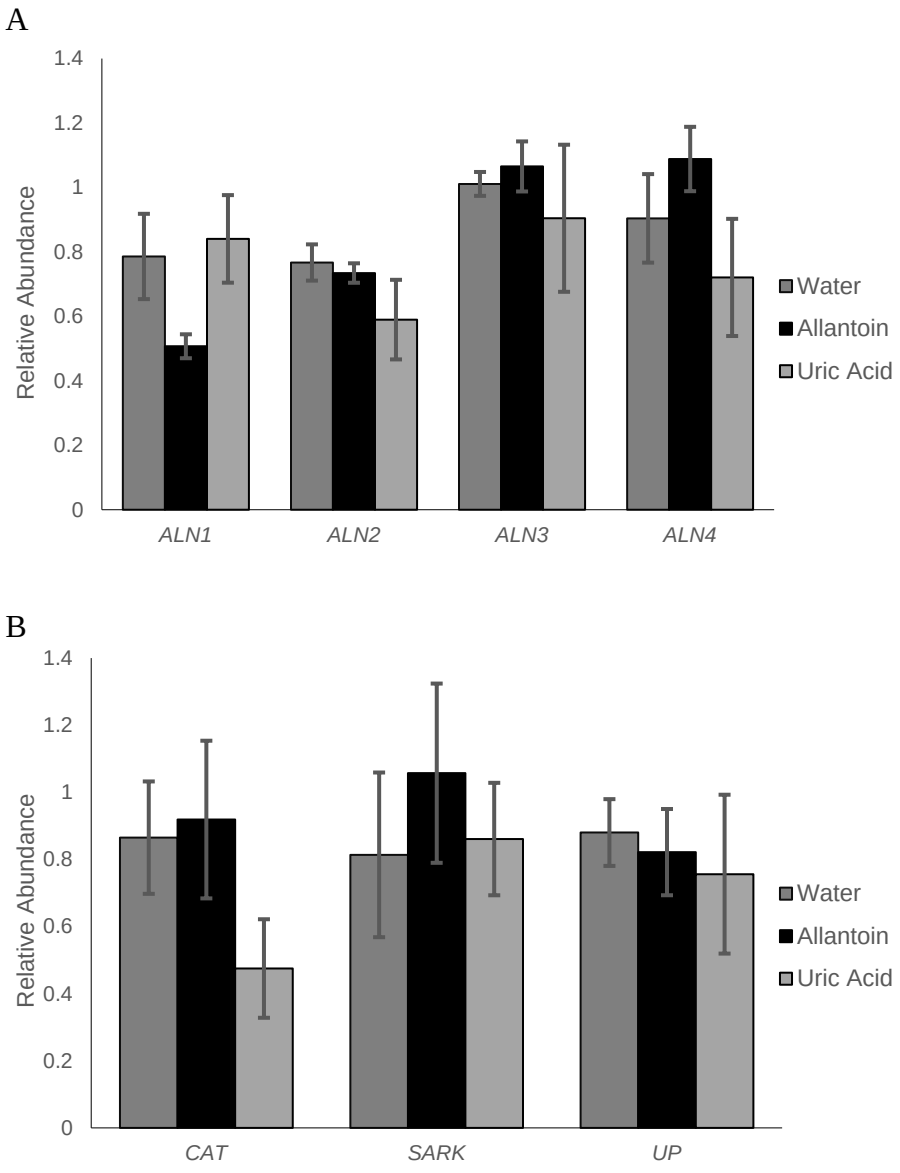


Figure 3.19: Expression of genes after treatment with H_2O_2. Relative transcript abundance of *ALN1-4* (A) and catalase (*CAT*) (B), a senescence-induced receptor-like kinase (*SARK*) and ureide permease (*UP*) in leaf disks pre-treated with water, allantoin or uric acid and treated with H_2O_2. RT-PCR was completed and electrophoresis gels were quantified. Data presented are averages of three replicates of treatment values normalized against controls ± standard error. A fragment of 18S rRNA was used as the quantitative control.

pre-conditioned plant tissue had decreased expression of *CAT* compared to control. No difference existed between pre-conditioning solutions for any of these genes (Figure 3.19 B).

3.5. Effect of ureides on endogenous ROS

To understand if ureides have the same effect on endogenous ROS as they do to exogenous ROS, the same experiments (Figure 3.11) were completed, substituting MV for H_2O_2, to stimulate ROS production inside the cells. Ureide content, ROS content, allantoinase activity, protein content and lipid peroxide content were all determined in the same manner. Control leaves were handled identically between the exogenous and the endogenous ROS treatment, therefore data for controls were combined and control values reported are the same for both experiments.

3.5.1. Cell death is reduced by pre-conditioning with allantoin and treatment with MV alters ureide and ROS content

Similar to the exogenous ROS treatment, leaves pre-conditioned with water and treated with MV contained less allantoin (3.0 nmol/leaf disk) than the leaves that did not receive the treatment (9.4 nmol/leaf disk). Uric acid levels were unchanged with and without treatment with MV. No allantoate was detected (Figure 3.20; Table B-3).

Allantoin content was greater in control leaves pre-conditioned with allantoin (40.6 nmol/leaf disk) than it was in the corresponding MV-treated leaf disks (13.1 nmol/leaf disk). Similar to the H_2O_2 treatment, uric acid was higher in the allantoin pre-conditioned leaves treated with MV (0.5 nmol/leaf disk) than the control leaves (0.2 nmol/leaf disk), however this relationship was not significant. Also, allantoate content in allantoin pre-conditioned leaves was significantly less in the MV-treated leaf disks (31.0 nmol/leaf disk) than in the control disks (98.9 nmol/ leaf disk) (Figure 3.20).

Uric acid pre-conditioned leaf disks contained an average of 33.9 nmol/leaf disk of uric acid and 10.9 nmol/leaf disk of allantoin after 48 hours. In the MV-treated leaf disks uric acid decreased to 20.44 nmol/leaf disk and allantoin decreased to 3.2 nmol/leaf disk, however

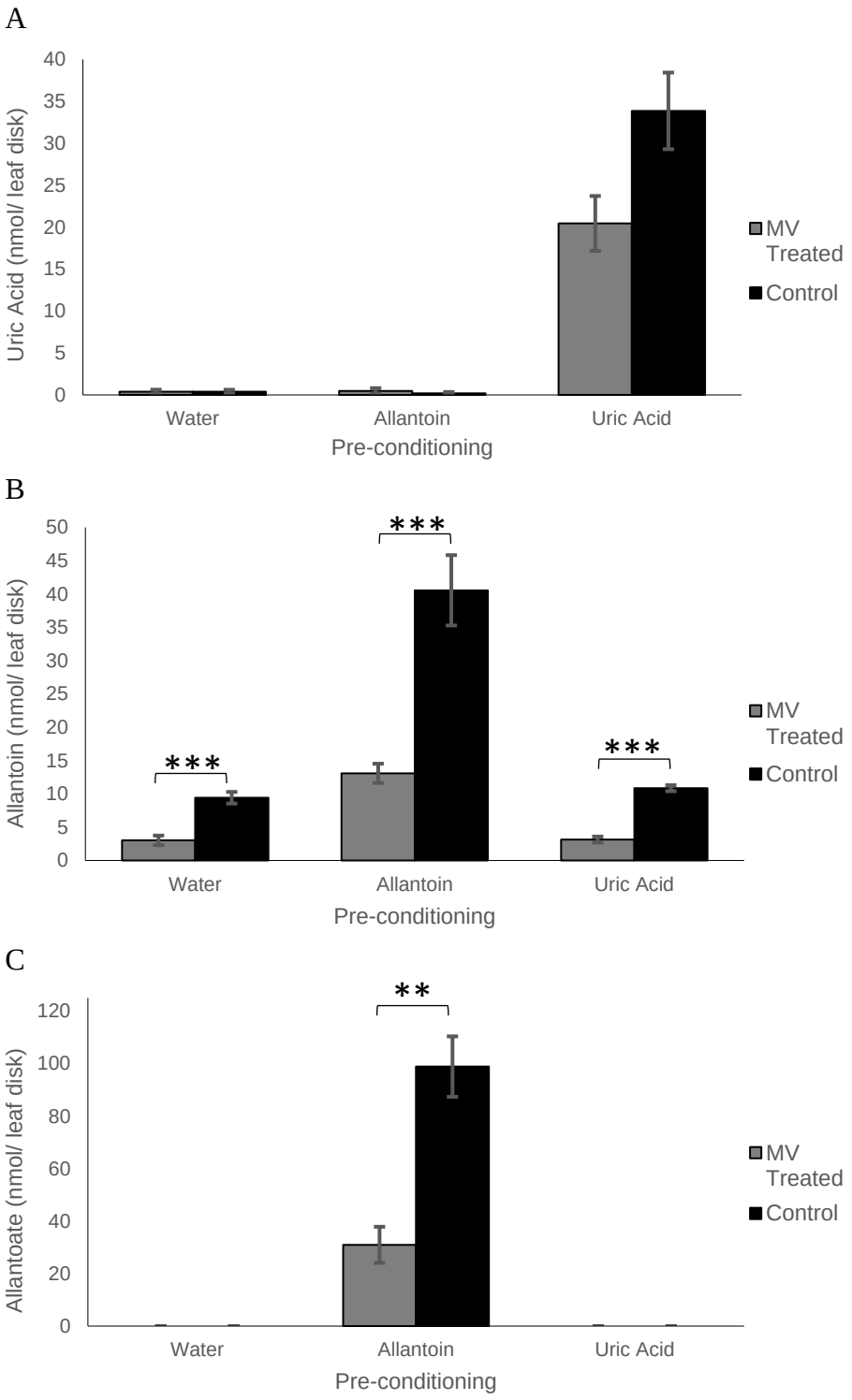


Figure 3.20: Ureide content in leaves after treatment with MV. Uric acid (A), allantoin (B), and allantoate (C) quantity in nitrogen-fertilized leaves after 24 hours of conditioning in water, allantoin and uric acid then 24 hours with endogenous ROS treatment (MV). Controls were spiked with distilled water. Averages of 4-6 replicates are depicted with error bars depicting standard error. Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

the difference in uric acid content was not significant. No allantoate was detected in the leaves (Figure 3.20).

Treatment with MV decreased the average O_2^- and H_2O_2 content, however the effect of treatment was not statistically different, determined by a two-way ANOVA (Table A-10 and A-12). No difference between among the pre-conditioning solutions was significant. Also, no pair differences were significant (Figure 3.21).

Cell death was induced by treatment with MV (Figure 3.22). As mentioned in section 3.4.1, no difference exists between the cell deaths of the leaf tissue in the different pre-conditioned controls. However, differences based on the pre-conditioning solutions occurred after treatment with MV. Leaves pre-conditioned with allantoin had the lowest percentage of cell death (24.9%). Water pre-conditioned tissue had greater cell death (31.5%) than the tissue pre-conditioned in allantoin solution. In contrast to exogenous ROS, leaves treated with MV after being pre-conditioned in uric acid had a greater amount of cell death (36.2%) compared to the leaf disks pre-conditioned in allantoin solution (Figure 3.22). The effect of treatment and pre-conditioning solution was significant as well as the interaction between the pre-conditioning solution and treatment, determined by two-way ANOVA (Table A-14).

3.5.2. Allantoinase activity and protein content decrease after treatment with MV and lipid peroxides increase

Allantoinase activity was analyzed in leaf tissue treated as previously described. Interestingly, although allantoinase activity decreased after treatment, the decrease was only significant in the allantoin and the uric acid pre-conditioned tissue (Figure 3.23). Allantoinase activity was lower after treatment in the water pre-conditioned tissue (0.064 nkat/ mg protein) than after treatment in the allantoin pre-conditioned tissue (0.070 nkat/ mg protein) although not significantly. However, allantoinase in the tissue pre-conditioned with uric acid (0.39 nkat/ mg protein) was significantly lower than the allantoinase activity in the allantoin pre-conditioned tissue (Figure 3.23). Both MV treatment and pre-condition type had a significant effect on allantoinase activity, determined by two-way ANOVA (Table A-15).

Similar to the exogenous ROS treatment, protein content decreased in leaf tissue after

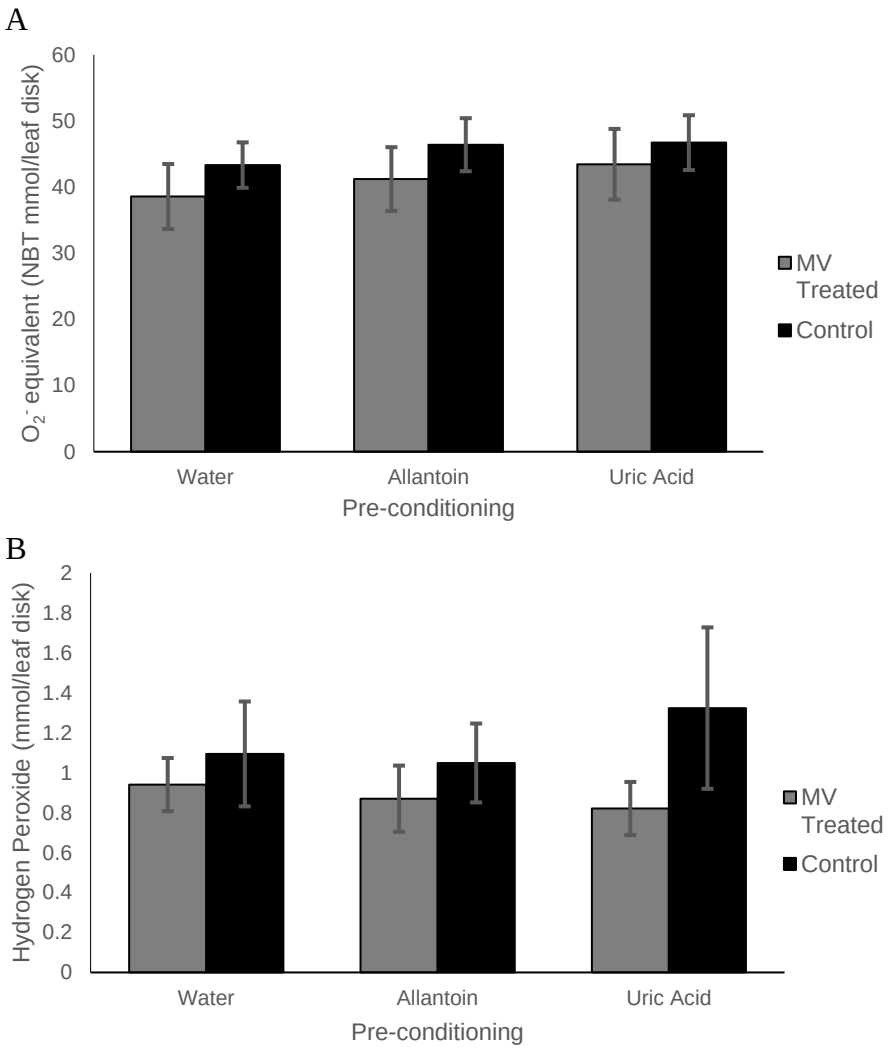


Figure 3.21: ROS content in leaves after treatment with MV. O_2^- (A) and H_2O_2 (B) content in soybean leaves pre-conditioned with water, allantoin or uric acid and treated with MV. Data represents averages of 7-9 replicates for O_2^- measurements and 5-7 replicates for H_2O_2 measurements. Error bars represent standard error.

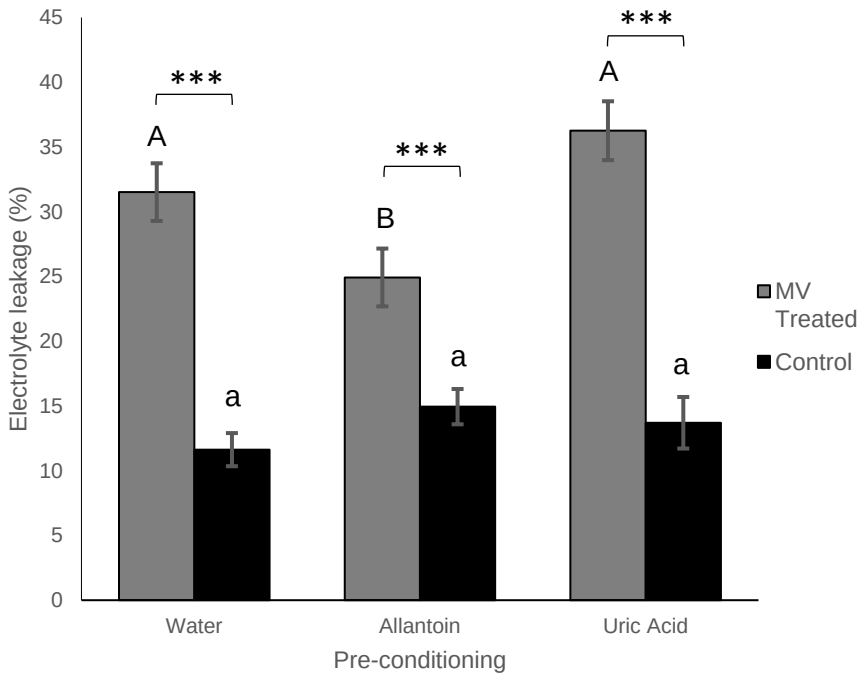


Figure 3.22: Cell death in soybean leaves after treatment with MV. Cell death in soybean leaf disks pre-conditioned with water, allantoin or uric acid and treated with MV. Data presented are the average of 20 independent replicates for controls and 13-14 independent replicates for treatment samples ± standard error. Upper and lower case letters indicate differences within treatment or control. Asterisks indicate difference due to treatment with MV determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

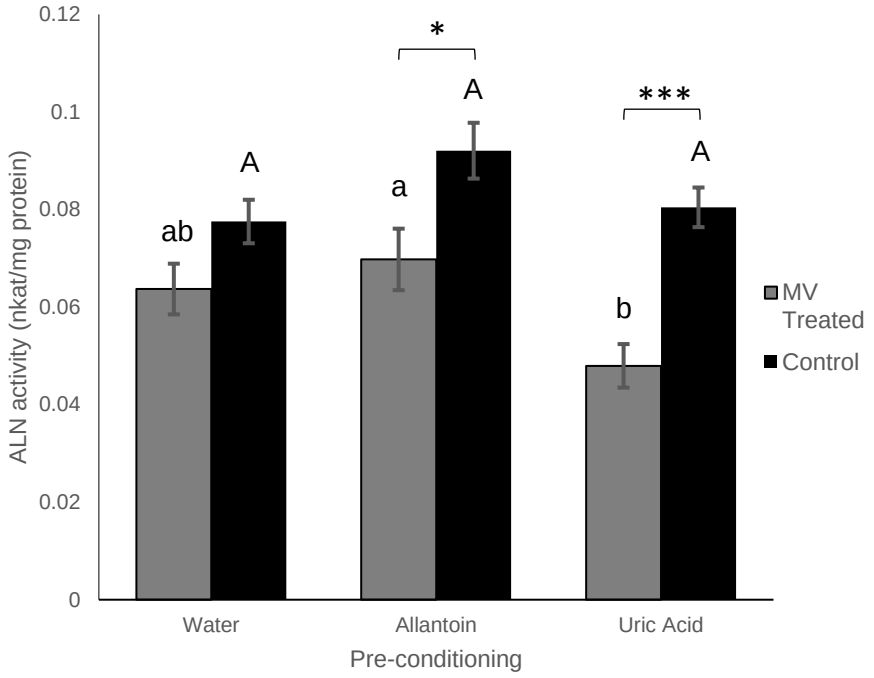


Figure 3.23: Allantoinase specific activity after treatment with MV. Allantoinase activity in soybean leaf disks after pre-conditioning with water, allantoin and uric acid and treatment with MV. Data presented are the averages of 7 independent replicates for controls and 5 independent replicates for treatment samples ± standard error. Upper and lower case letters indicate differences within the treatment or the control ($p < 0.05$), determined by Tukey's test. Asterisks indicate difference due to treatment with MV determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

treatment with MV. The decrease, however, was not significant in the allantoin pre-conditioned leaf disks compared to allantoin pre-treated controls (Table A-17). Pre-conditioning solutions did not have a significant effect on the decrease, however the average protein in the samples pre-conditioned in allantoin (8.9 mg) was greater than both water (7.5 mg) and uric acid (7.9 g) pre-conditioned tissue (Figure 3.24).

Lipid peroxides were measured after treatment with MV. After treatment with MV, lipid peroxides ranged from an average of 0.32 umol MDA/g protein in allantoin pre-conditioned tissue to 0.38 umol MDA/g protein in uric acid pre-conditioned tissue (Figure 3.25) compared to 0.25 to 0.27 umol MDA/g, but these were not significantly different ($p < 0.05$) (Table A-18). Overall lipid peroxidation was lower after treatment with MV than what was observed after treatment with H_2O_2.

3.5.3. Transcriptional changes due to MV treatment

Gene expression was examined after treatment with MV. Visually, it appeared the expression of all the genes decreased after treatment, with exception of *ALN3* and *ALN4* (Figure 3.26). Differences between pre-conditioning solutions were not immediately obvious.

MV treatment caused changes in gene expression similar to H_2O_2 treatment, with a few exceptions. *ALN1* decreased in leaf tissue pre-conditioned with water and allantoin. In contrast to treatment with H_2O_2, treatment with MV did not decrease expression of *ALN2*. *ALN4* also had no changes in expression. Interestingly, *ALN3* did not change in tissue pre-conditioned with water but increased after MV treatment for every replicate after pre-treatment with allantoin or uric acid (Figure 3.27 A). However, like previous experiments, results were variable and no statistical differences were determined under three replicates. *CAT* expression decreased after MV treatment, but this result was only significant in samples pre-conditioned with water and allantoin. *SARK* expression also decreased in samples pre-treated with allantoin and uric acid. The only significant decrease occurred in tissue pre-conditioned with allantoin, in regards to *UP* expression, although all three replicates also decreased in tissue pre-treated with uric acid (Figure 3.27 B). When analyzed by one-way ANOVA, no difference existed between pre-conditioning solutions for any

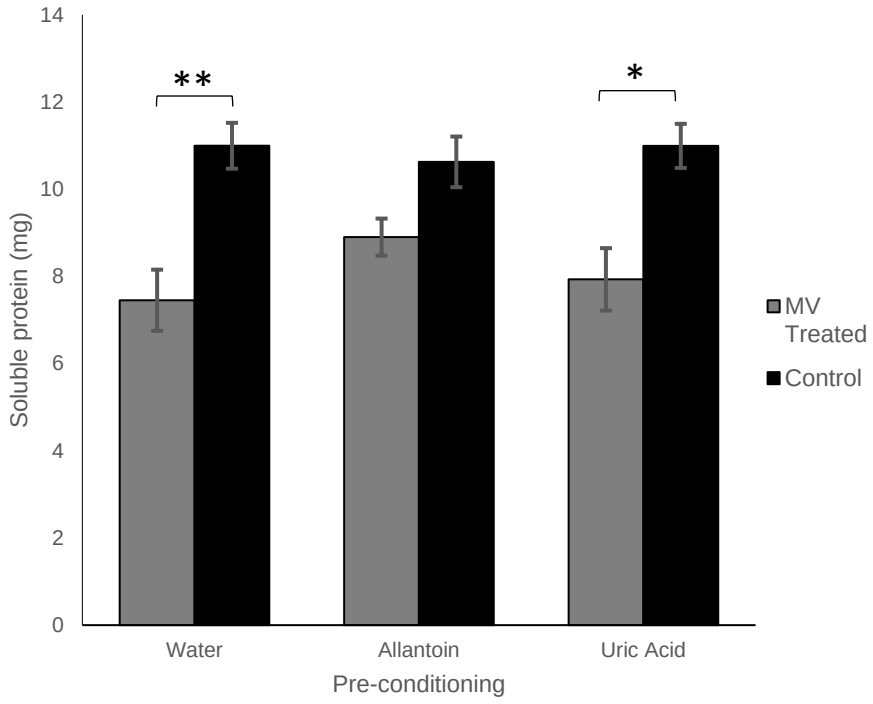


Figure 3.24: **Protein content after treatment with MV.** Soluble protein in soybean leaf disks after pre-conditioning with water, allantoin and uric acid and treatment with MV. Data presented are the means of 7 independent replicates for controls and 6 independent replicates for treatment samples ± standard error. Asterisks indicate significance between pairs determined by targeted Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

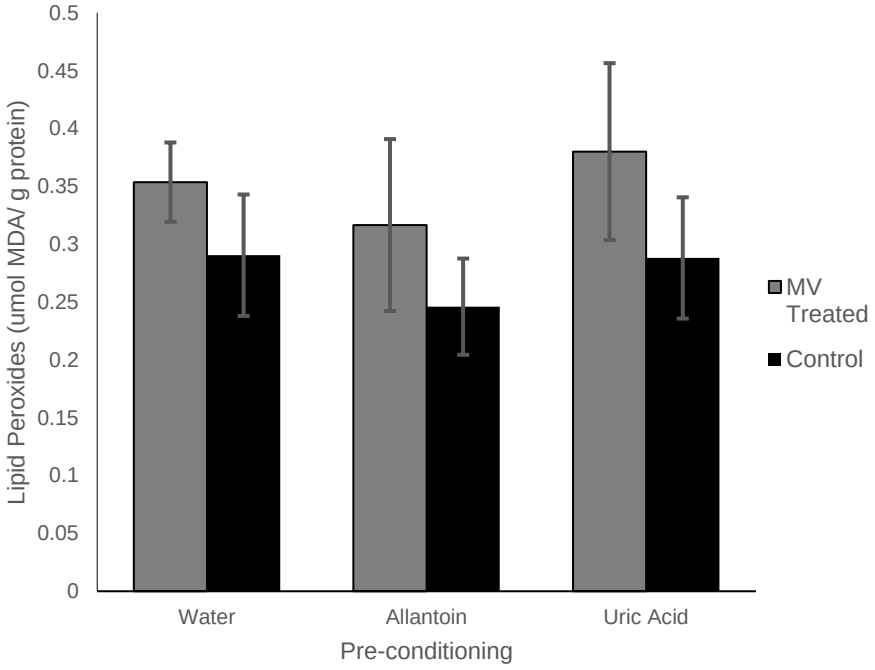


Figure 3.25: Lipid peroxides in leaves after treatment with MV. Lipid peroxides per mg of protein in soybean leaf disks pre-conditioned with water, allantoin, or uric acid and treated with MV. Data shown are the mean of four replicates ± standard error.

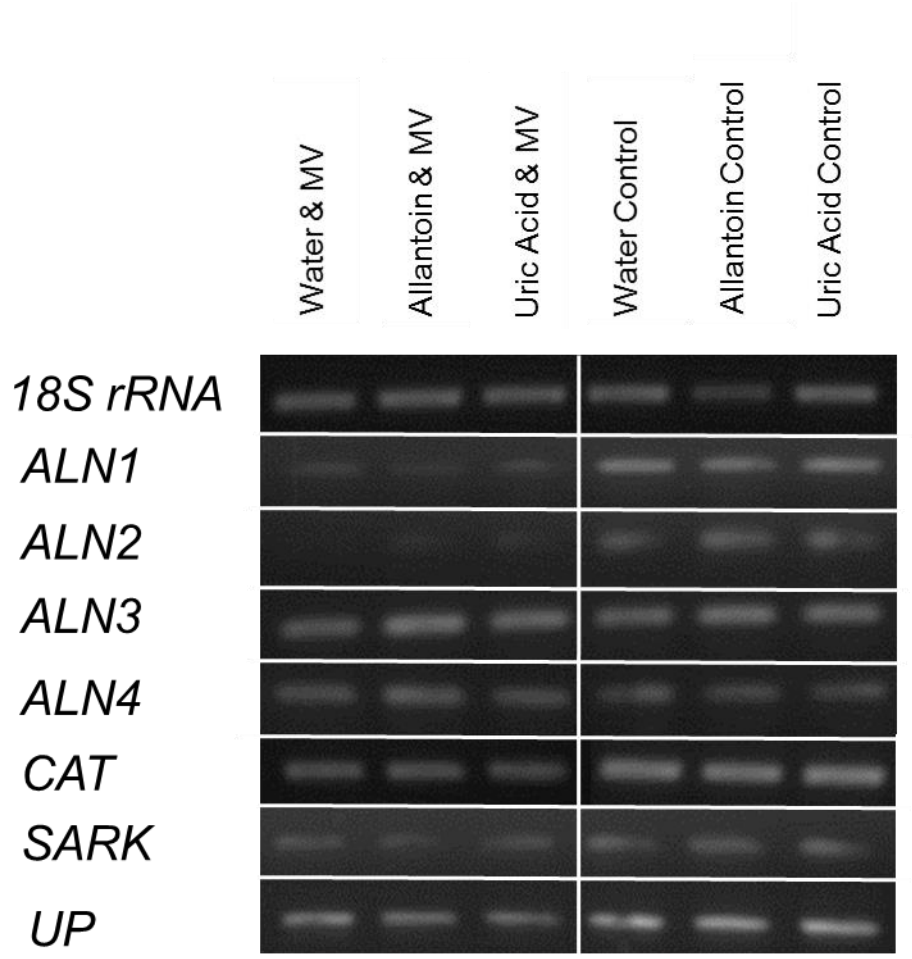


Figure 3.26: Gene expression in soybean leaf tissue after treatment with MV. Expression of genes in leaf disks of fully expanded young soybean leaves after pre-conditioning with water, allantoin or uric acid and treatment with MV. *SARK*: senescence associated receptor-like kinase; *ALN*: allantoinase; *CAT*: catalase; *UP*: ureide permease. Reference gene is a 209bp fragment of soybean 18S rRNA. Image shown is representative of three independent replicates analyzed.

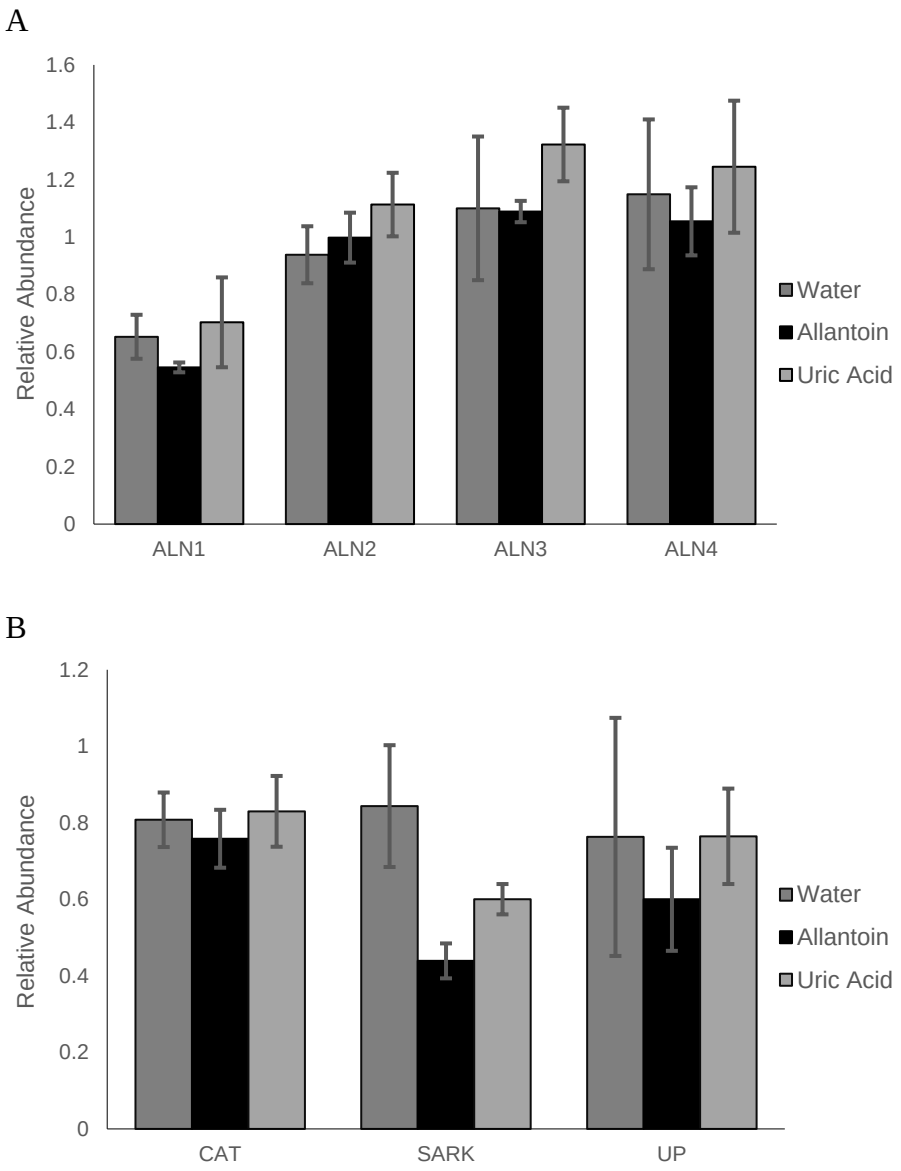


Figure 3.27: Gene expression in soybean leaf tissue after treatment with MV. Relative transcript abundance of *ALN1-4* (A) and catalase (*CAT*) (B), a senescence-induced receptor-like kinase (*SARK*) and ureide permease (*UP*) in leaf disks pre-treated with water, allantoin or uric acid and treated with MV. RT-PCR was completed and electrophoresis gels were quantified. Data presented are the mean of three replicates of treatment values normalized against controls ±standard error. A fragment of 18S rRNA was used as the quantitative control.

of these genes. Average *SARK* expression decreased to a greater extent in leaf tissue pre-conditioned with allantoin, but it was not significant.

3.6. Are uric acid and allantoin antioxidants?

3.6.1. Uric acid scavenges H_2O_2

Since reports suggest possible antioxidant effects of uric acid and allantoin, along with observations in the leaves from this experiment, I tested whether the ureides were acting as antioxidants under the experimental conditions used for the leaf disk experiment. The pre-conditioning solutions without leaf disks had H_2O_2 added. No leaf tissue came into contact with the solution, therefore the results represent the chemical breakdown of H_2O_2 due to each pre-treatment. H_2O_2 quantity was assayed 90 minutes after it was added to the pre-conditioning solution and 24 hours after it was added to the pre-conditioning solution (parallel to the main experiment). At both times the uric acid pre-conditioning solution containing significantly less H_2O_2 than the allantoin or the water pre-conditioning solution, determined Tukey's test ($p < 0.001$). No statistical differences existed in H_2O_2 content between the water pre-conditioning solution and the allantoin pre-conditioning solution, however after 24 hours the H_2O_2 quantity in both of these solutions decreased from the initial concentration (Figure 3.28).

3.6.2. Allantoin decreases ROS in solution when leaf tissue is present

Because of the lack of reaction between allantoin and H_2O_2, the effect of leaf tissue on the solution they were floated in was tested. In the H_2O_2 treatment, the water pre-conditioning solution contained the highest average O_2^- for both the control (20.1 mM NBT) and for the treated tissue (94.2 mM NBT), however these were not statistically greater than in the allantoin pre-conditioning solution or the uric acid pre-conditioning solution (Figure 3.29 A). After treatment with MV, the uric acid pre-conditioning solution contained the largest quantity of O_2^- (105 mM NBT). However, again, there was no statistical difference between the pre-conditioning solutions after MV

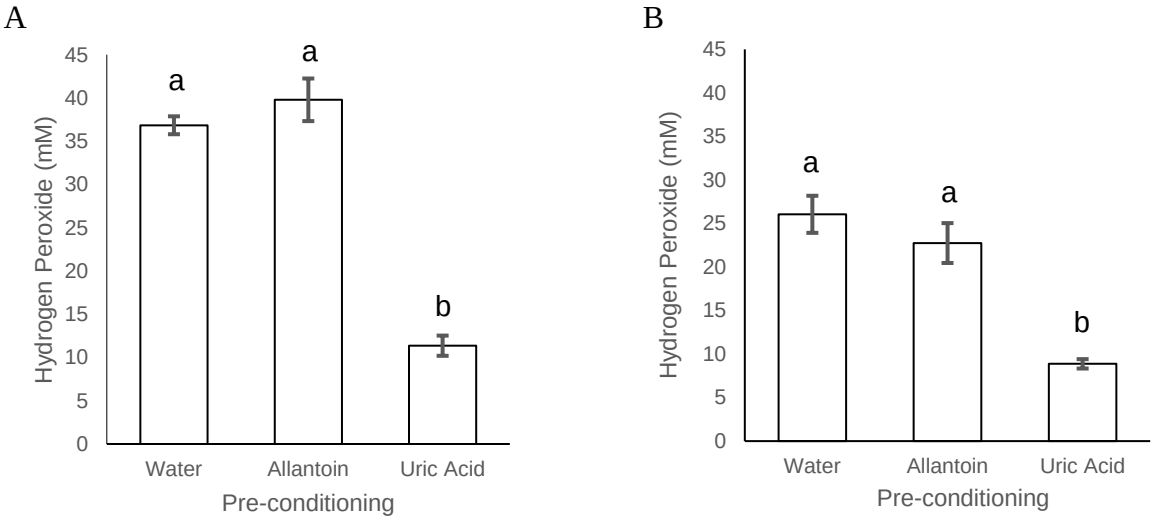


Figure 3.28: H_2O_2 content in solutions without leaves after treatment with H_2O_2. Hydrogen peroxide content in solutions of water, allantoin of uric acid 90 minutes after the addition of H_2O_2 (A) and 24 hours after the addition of H_2O_2 (48 hours after pre-treatment) (B). Data presented are averages of 7 and 8 independent replicates for A and B, respectively, ± standard error. Lower case letters indicate differences within the pre-conditioning solution, determined by a post hoc Tukey's test.

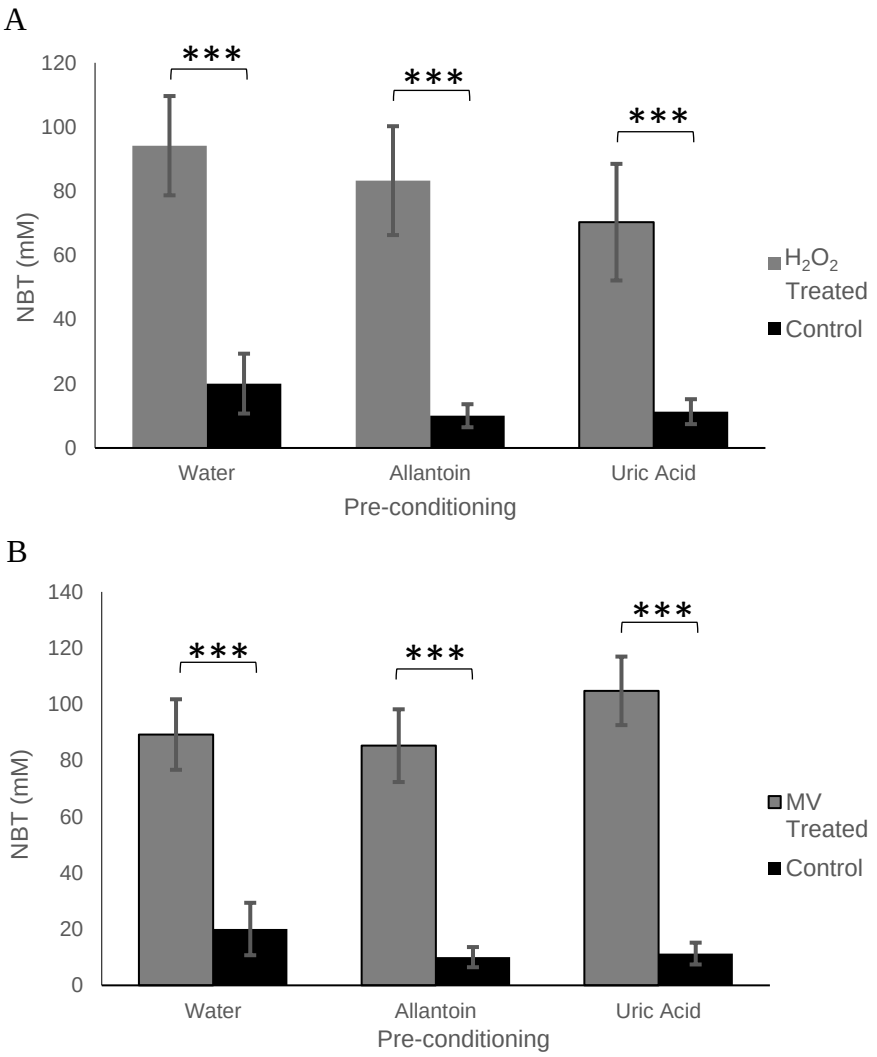


Figure 3.29: Superoxide content after treatment with H_2O_2 and MV. Superoxide content (represented by NBT) in pre-conditioning solutions after treatment with H_2O_2 (A) and MV (B). Solutions contained soybean leaf disks. Data presented are averages of 17 replicates for controls and 12 for treated ± standard error. Controls in A and B are identical values. Asterisks indicate difference due to treatment determined with Student's t-tests (p-value: <0.05*, <0.01**, <0.001***).

treatment (Figure 3.29 B). After treatment with H_2O_2 or MV, O_2^- increased in the presence of the leaf tissue, but the type of pre-conditioning solution did not have an effect on the results and there was no interaction between the pre-conditioning solution and the treatment using two-way ANOVA (Table A-11).

H_2O_2 was also measured in the pre-conditioning solution that contained leaf tissue after treatment with MV and H_2O_2. After treatment with H_2O_2, the average quantity of H_2O_2 significantly increased in the water pre-conditioning solution from 11.8 mM to 30.4 mM (Figure 3.30 A). A significant increase also occurred in the uric acid pre-conditioning solution, as average H_2O_2 grew from 16.9mM to 30.9mM. Interestingly, no significant increase in H_2O_2 quantity occurred in the allantoin pre-conditioning solution (21.4 mM in the control and 23.7 mM in the treated solution), partially because of the increase of H_2O_2 in the allantoin pre-treated control. After treatment with MV, the amount of H_2O_2 in the water pre-conditioning solution increased from 11.8mM to 32.4 mM, a similar relationship to what occurred in the H_2O_2 treatment (Figure 3.30 B). Average H_2O_2 went up in the uric acid pre-conditioning solution, from 16.9 mM to 24.3 mM, but the increase was not significant. Determined by a two-way ANOVA, an interaction between pre-conditioning solution and treatments occurred in both treatments (Table A-13).

Unexpectedly, comparing control solutions, there was more H_2O_2 when allantoin or uric acid were present (Figure 3.30). The water pre-conditioning solution only contained 11.8mM of H_2O_2, whereas the allantoin pre-conditioning solution contained a significantly greater quantity of 21.4mM. The uric acid pre-conditioning solution may have caused the leaf to evolve H_2O_2 (16.9mM) but was not significantly different from the other two pre-conditioning solutions.

3.6.3. Ureides in the supernatant change after treatment with H_2O_2 or MV

The pre-conditioning solution in which the leaves were floated in was analyzed to determine the interaction between the ureide concentration in the leaves and in the solution. Analysis was also completed on supernatant that was treated in parallel to the experiment but did not contain leaf tissue.

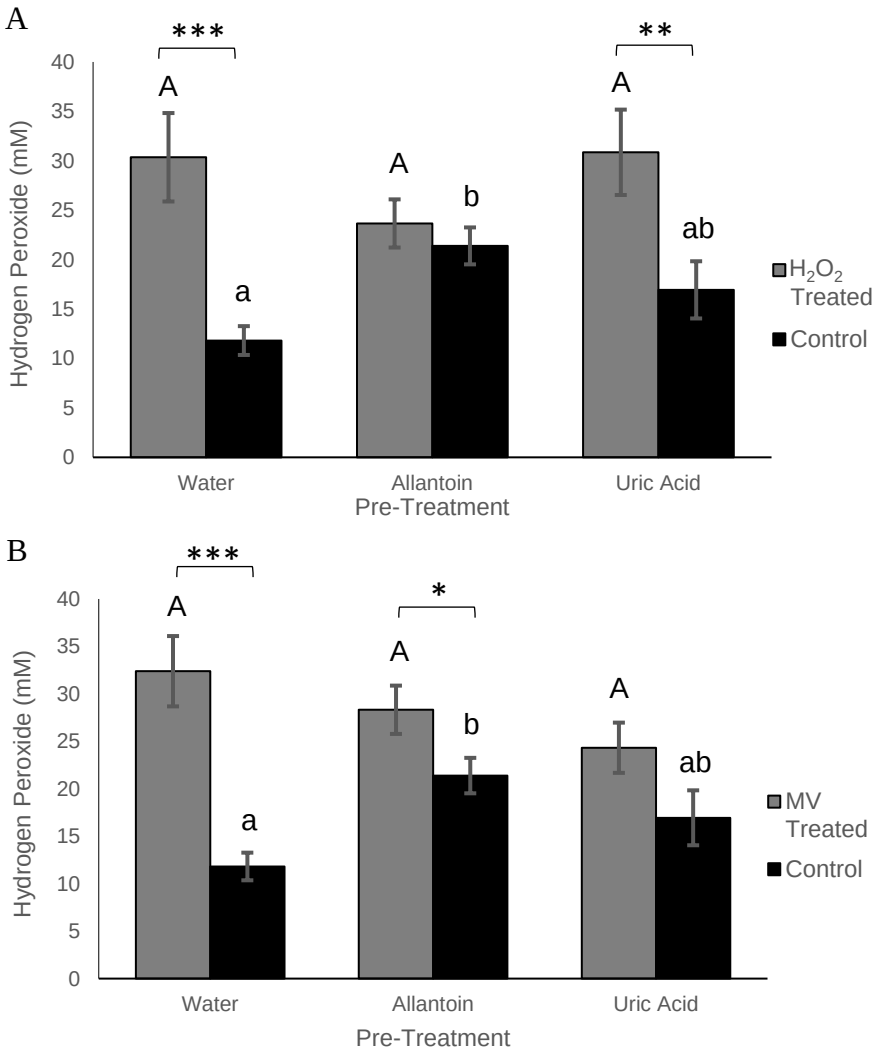


Figure 3.30: H_2O_2 content after treatment with H_2O_2 and MV. H_2O_2 concentration in the pre-conditioning solutions that contained leaves after treatment with H_2O_2 (A) and MV (B). Data presented are averages of 17 replicates for controls and 12 for treated ± standard error. Controls in A and B are identical values. Upper and lower case letters indicate differences within treatment or control. Asterisks indicate difference due to treatment determined with a Student's t-test (p-value: <0.05*, <0.01**, <0.001***).

Ureide content in the pre-conditioning solutions was determined in the treated and control solution to confirm uptake into the leaf disks. Virtually no ureides were measured in the water pre-conditioning solution in the absence of H_2O_2 or MV and trace amounts accumulated after 24 hours with either compound (Figures 3.31 and 3.32 A). In the both allantoin and uric acid pre-conditioning solutions there was evidence of uptake of either compound over the assay period, however more allantoin was present in the solution after 24 hours with either H_2O_2 or MV compared to the control solution (Figures 3.31 B and 3.32 B), likely indicating decreased uptake. Substantial allantoate was also present in the allantoin pre-treatments. Interestingly, although the amount of allantoin was similar between H_2O_2 and MV solutions the amount of allantoate in the H_2O_2 treated solution was almost half that of what was quantified in the MV-treated solution (Table B-4). Likely due to slight evaporation the concentration of ureides was higher in solution than expected, however the changes in solution amounts were not determined and therefore evaporation cannot be confirmed.

Two different reactions were observed with the uric acid pre-conditioning solution. Samples treated with H_2O_2 contained less uric acid than the control (Figure 3.31 C), whereas solutions treated with MV contained more uric acid than the control (Figure 3.32 C), however neither difference was significant. Allantoin and allantoate quantity was greater in the H_2O_2-treated solution than the control solution, but only the difference in allantoin was significant. Allantoin and allantoate on average were also greater in the MV-treated solution than the control solution, however neither difference was significant (Figure 3.31 and 3.32 C; Table B-4).

To determine direct chemical interactions between ureides and ROS, ureide content of the pre-conditioning solutions was measured in the absence of leaf tissue after treatment with H_2O_2 or MV. In the absence of leaf tissue, allantoin content in the allantoin pre-conditioning solution did not change after treatment with either MV or H_2O_2 (Table B-4). Similarly, uric acid content did not decrease under treatment with MV when leaf tissue was not present. Interestingly, uric acid did, however, decrease by approximately 10% when treated with H_2O_2 in comparison with the control and allantoin was evolved. This data support previous findings that indicate uric acid as a possible water-soluble physiological antioxidant (Simic and Jovanovic, 1989). No ureides were measured in the water pre-conditioning solution without leaves after either treatment (Table B-4).

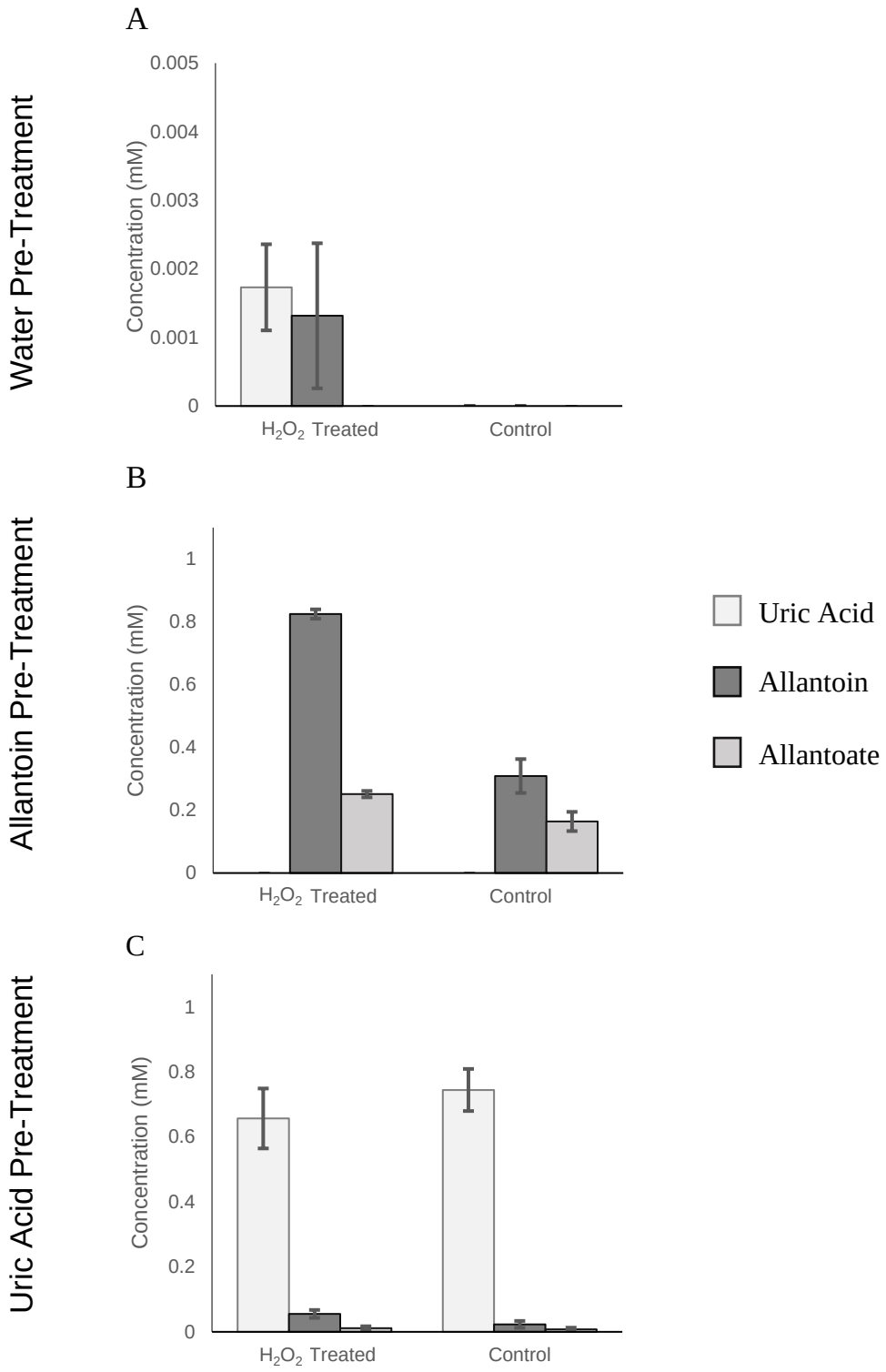


Figure 3:31: Ureide content in pre-conditioning solutions after treatment with H_2O_2. Ureide quantity in the pre-conditioning solution that contained leaf disks. Pre-conditioning solutions contained either water (A), allantoin (B), or uric acid (C). Analysis was completed after treatment with H_2O_2. Data presented represent averages of 6-11 independent replicates ± standard error.

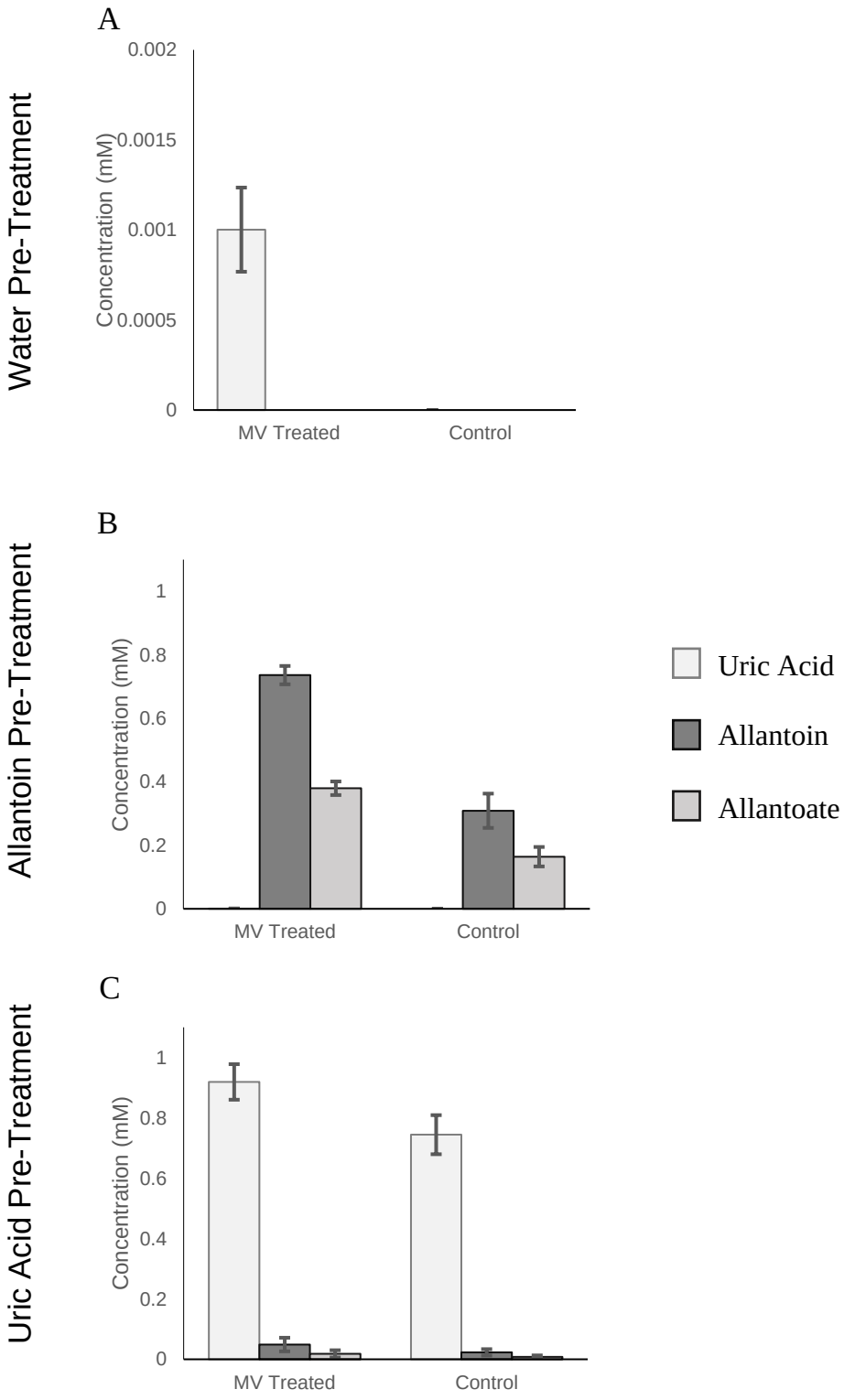


Figure 3.32: Ureide content in pre-conditioning solutions after treatment with MV. Ureide quantity in the pre-conditioning solution that contained leaf disks. Pre-conditioning solutions contained either water (A), allantoin (B), or uric acid (C). Analysis was completed after treatment with MV. Data presented averages of 6-11 independent replicates ± standard error.

3.6.4. Summary of isolated leak disk and oxidative stress results

Leaf disks took up the ureide provided in the pre-conditioning solution and metabolized the ureide. I hypothesized that uric acid and allantoin scavenge the excess ROS produced during abiotic stress. Uric acid reacted chemically with H_2O_2, but allantoin did not. Nevertheless, allantoin decreased the accumulation of H_2O_2 in the solution after treatment with H_2O_2 and MV when the leaf tissue was present. However, addition of allantoin into a solution containing a leaf increased the H_2O_2 in the absence of exogenous H_2O_2 or MV. The amount of O_2^- and H_2O_2 in the leaves did not differ due to the ureide pre-conditioning.

The hypothesis that increased ureides have a beneficial effect on the cell and will mitigate tissue damage in response to ROS was supported by this research. Both uric acid and allantoin decreased cell death due to H_2O_2 and allantoin decreased cell death due to MV. Lipid peroxides did not differ due to allantoin or uric acid. Allantoin also may have decreased the degradation of the soluble protein after treatment with MV.

3.7. Differences in tepary bean response to increased ROS after application of MV

To determine whether ureide accumulation during abiotic stress is under genetic control, I examined whether differences in patterns of ureide accumulation and responses to endogenous ROS exist among tepary bean genotypes. Leaf disks sampled from 108 genotypes grown in a growth chamber were bathed in a 0.5 mM MV solution for 24 hours. Uric acid, allantoin, and allantoate were measured in both treated leaf disks and control leaf disks that were bathed in distilled water. The majority of genotypes contained more ureides in the MV treated leaf tissue than the control tissue, in varying amounts (Figure 3.33). However, the MV treated leaf disks of

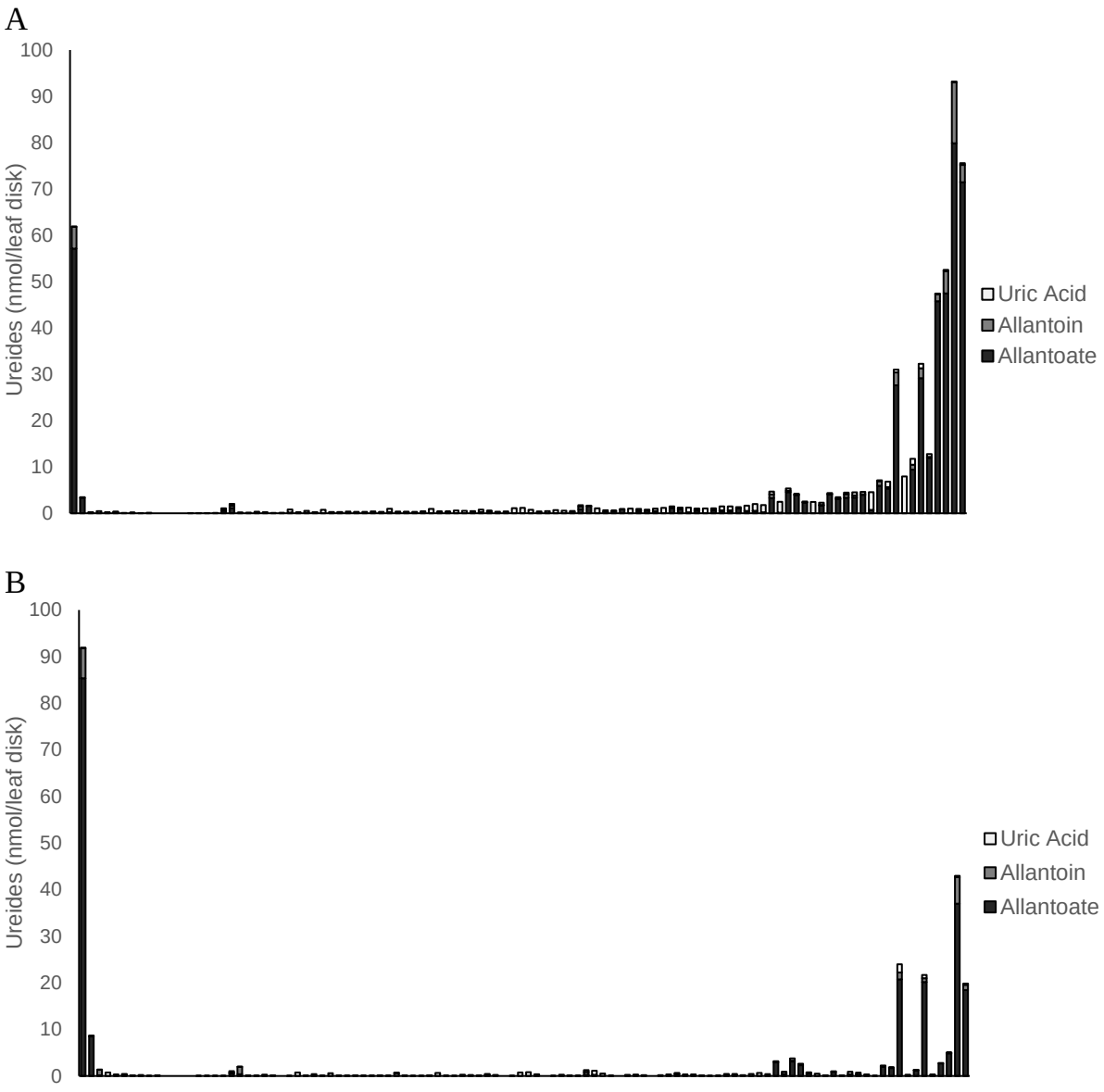


Figure 3.33: Survey of tepary bean genotypes in response to MV treatment. Ureide quantity was analyzed in 108 tepary bean genotypes after being bathed in a solution of methyl viologen (A) to induce ROS production or distilled water (B). Genotypes are ordered by the difference between the treated and the control for total ureides, from small (including negative difference) to large.

several genotypes contained less uric acid, allantoin, and allantoate than the control. The largest difference in any ureide appeared to be in allantoate content, observed either as an increase or decrease, depending on genotype. Differences in allantoin and uric acid were apparent in some genotypes but response between genotypes varied. Twenty six genotypes were selected based on differing responses in ureide accumulation for a replicated experiment. Table B-5 provides a list of genotypes and changes in ureide quantity.

26 tepary beans were selected from the large subset for replication (Table B-5). A common bean, NY5-161, and two additional tepary genotypes, PI 430219 and W6 15578, were added to the experiment, because NY5-161 and W6 15578 were the parents of the interspecific introgression lines to be studied later and PI 430219 was of interest to the breeding program. Because the plants in the original experiment were donated for the purposes of the preliminary experiment, data from the original experiment was not included into the analysis of the subsequent replications as growth conditions could not be guaranteed to be the same. A similar trend to the preliminary experiment was observed, with the majority of the accessions' leaves demonstrating increased ureide content in response to MV, but several accessions also decreasing. Allantoin and allantoate content were significantly different between the treated and control leaf disks, but uric acid was not, determined by individual two-way ANOVAs for each compound (Table A-19). After calculating the difference of total ureides (uric acid, allantoin and allantoate) between treated tissue and control tissue, targeted t-tests indicated differences among genotypes, including G40302 accumulating less total ureides than all genotypes except G40173. Tepary accession G40173 accumulated less ureides than seven other accessions, including G40158A, G40038, G40036, G40141, G40021, G40066A and Mitla Black (Figure 3.34). The average difference between genotypes did not correlate between the replicated trial and the preliminary survey, however correlations between individual ureides exist (Table A-20). The preliminary experiment contained magnitude higher concentration of ureides compared to the sub-set experiment (Figures 3.33 and 3.34), possibly due to differences in growth conditions between the two experiments.

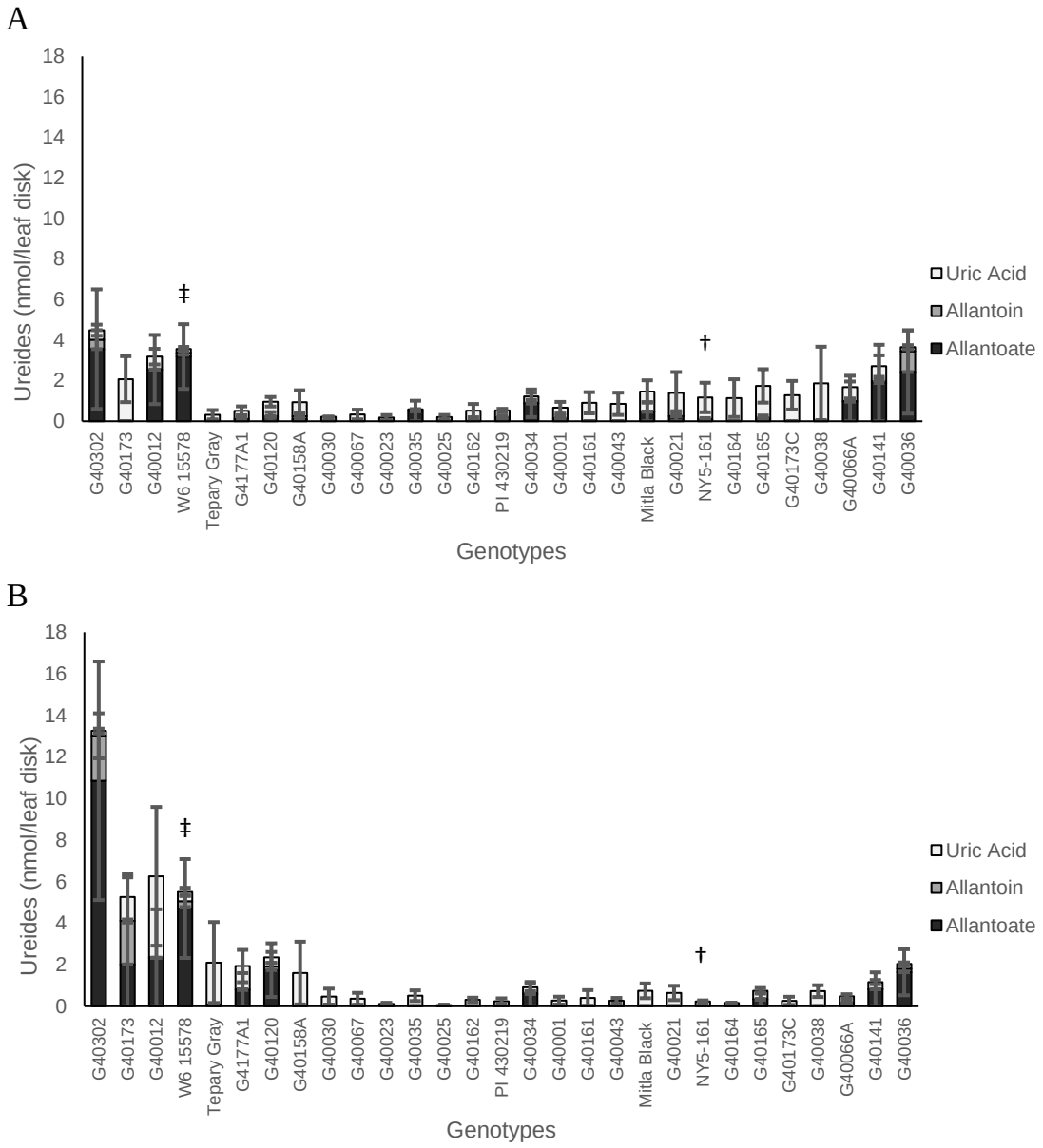


Figure 3.34: Subset of tepary beans and NY5-161 and the response to treatment with MV. After a large tepary bean survey (Figure 3.33) a subset of 26 tepary beans were selected for further analysis. An additional two tepary beans (W6 15578 and PI 430219) and one common bean (NY5-161) were added for examination. Ureide quantity in leaf disks floated in MV (treated) (A) and distilled water (control) (B) are depicted. Genotypes are ordered by the difference between the treated and the control, from small (including negative difference) to large. The means of three replicates is shown in the stacked bar graph ± standard error. NY5-161 is indicated by a dagger symbol (†) and W6 15578 is indicated with a double dagger symbol (‡).

3.8. Sub-zero temperature stress in tepary bean, common bean and interspecific introgression lines

To address the hypothesis that tepary bean can be used to transfer genes encoding abiotic stress tolerance traits to common bean, twelve interspecific introgression lines (including at least one from each independent hybrid back cross discussed in section 2.1), three tepary beans (Tepary Gold, Tepary White and W6 15578) and one common bean (NY5-161) were removed from standard conditions and placed in a cold chamber (7°C/5°C day/night temperature, 16hr/8hr day/night photoperiod) temperature for two days. Leaf disks were then cut from the leaves and inserted into microfuge tubes. Tubes were placed in a dark chilling bath and subjected to a sub-zero temperature stress as described in section 2.2.3.1. The content of O_2^- and H_2O_2 and the electrolyte leakage of the leaves were compared with control leaf disks which were kept in the dark in a cooler for the duration of the experiment. Due to lack of space in the cold bath, the population was randomly split into two for all three replicates before acclimation and analyzed on back to back days. Homogeneity of variance confirmed no block effects occurred due to the multiple-day analysis.

O_2^- and H_2O_2 content were analyzed in the tissue immediately after returning to room temperature and also 24 hours after removal from cold treatment and was compared with control values. O_2^- and H_2O_2 content increased in most genotypes immediately following cold treatment (Figure 3.35), however these increases were not significant. After 24 hours, there was also no significant difference among genotypes in O_2^- content, but there was a significant difference among post-stress H_2O_2 contents (Table A-21). Results of a post-hoc Tukey test indicate Tepary Gold has a higher H_2O_2 content than E-6-7, W6 15578, D-13-4Br, A-14-10. The change in O_2^- and H_2O_2 content were not statistically different between the parents, however the tepary parent (W6 15578) had higher average O_2^- immediately after the stress than the common bean parent (NY5-161). W6 15578 had a lower average change in O_2^- 24 hours after the stress and lower average change of H_2O_2 at both time points analyzed, compared to NY5-161, however none of these relationships were significant using a post hoc Tukey's test. The interspecific introgression lines demonstrated a range of changes in O_2^- and H_2O_2 content, with genotypes demonstrating average changes varying around and between the average changes of the parents.

Correlation analysis determined the change in H_2O_2 measured immediately after treatment was significantly positively correlated to the change in H_2O_2 measured 24 hours after treatment

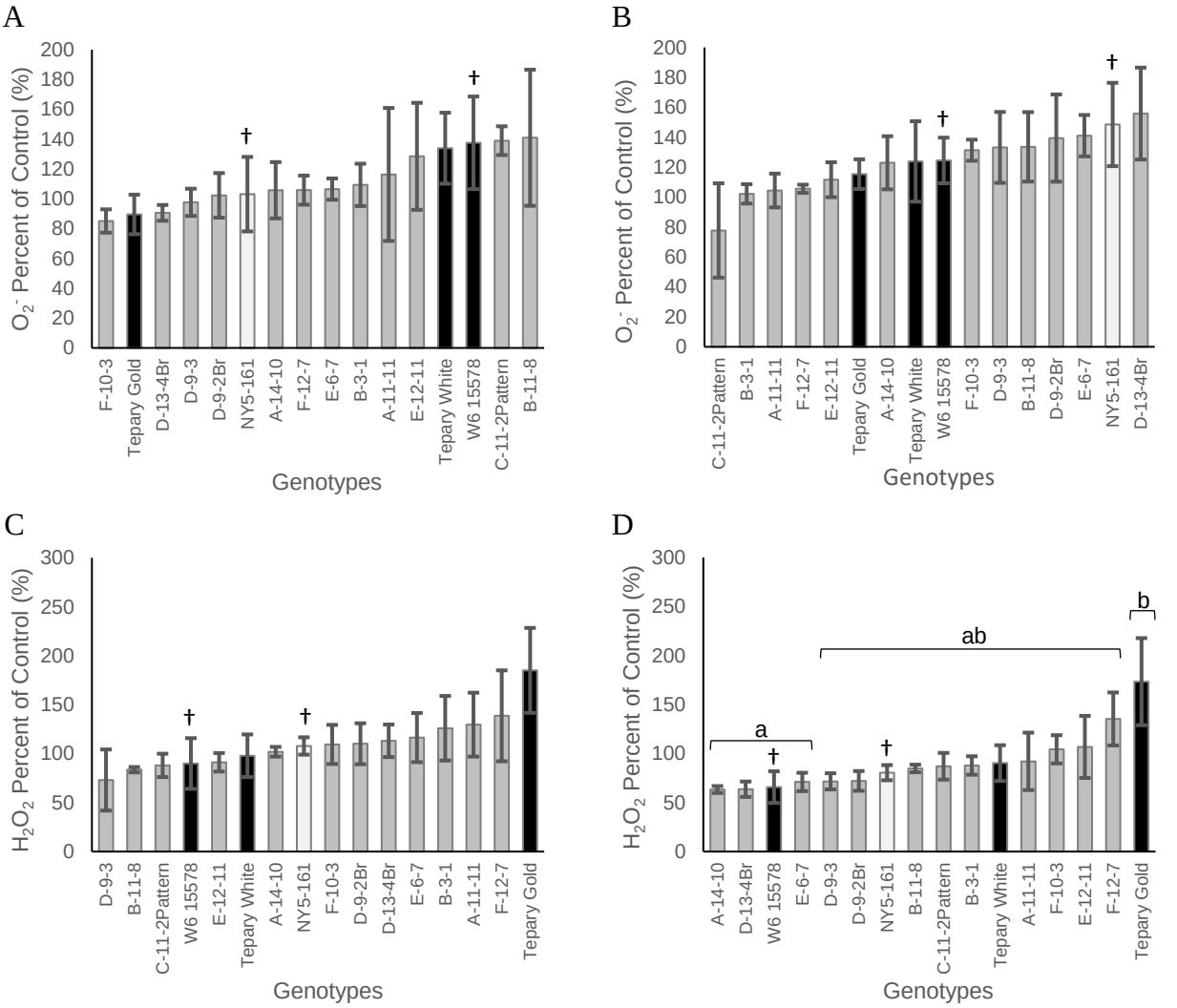


Figure 3.35: ROS content after a sub-zero temperature stress. Change in superoxide (A,B) and hydrogen peroxide (C,D) content in genotypes subjected to a freezing water bath. The change in ROS was measured immediately after treatment (A,C) and 24 hours after treatment (B,D). Three tepary beans (tepary gold, tepary white and W6 15578), one common bean (NY5-161) and twelve interspecific introgression lines were analyzed. Bars are the average of three independent replicates ± standard error. Any statistical differences observed between genotypes are denoted by lower case letters. The tepary beans are shown in black, the common bean in white and the interspecifics in gray. Parents are indicated with a dagger symbol (†).

(r= 0.354), but no correlation occurred for change in O_2^- content. Interestingly, a negative correlation existed between change in O_2^- and H_2O_2 content immediately after treatment (r= -0.290). A similar relationship was seen after 24 hours, but the correlation was not significant at $p< 0.05$ (Table A-23).

Electrolyte leakage was calculated immediately after sub-zero temperature treatment was applied to leaf disks to determine cell death (Figure 3.36). Cell death occurred after sub-zero temperature stress treatment, but the effect of genotypes and the interaction between the genotypes and stress were not significant, determined by a two-way ANOVA (Table A-22). When electrolyte leakage was analyzed as a percent of control, differences between genotypes became evident. A post hoc Tukey test determined W6 15578 had a lower change in electrolyte leakage than F-12-7, D-9-3, E-6-7 and D-9-2br.

The difference in cell death between the treated leaves and the control leaves was negatively correlated with the change in H_2O_2 immediately after treatment (r= -0.346) (Figure 3.37 A). The same relationship was seen with the change in H_2O_2 24 hours after treatment, but it was not significant. In contrast, a positive correlation existed between the difference in cell death and the change in O_2^- content in the leaves (r=0.472 and r=0.282, for instant O_2^- content and O_2^- content after 24 hours) (Figure 3.37 B). The change in O_2^- content and the percent of control cell death may have been positively correlated, but also not significant (Table A-23).

3.9. Sub-zero temperature field experiment

To address the hypothesis that genotypic differences in ureide content influences plant response to cold stress, three plantings of four replicates of 3 common beans, 20 tepary beans and 116 interspecific introgression lines of common bean and tepary bean were sown on sequential dates in 2013, 2014, and 2015 in an attempt to subject genotypes to a damaging sub-zero temperature at the seedling stage. Each planting was grown in a complete randomized block design. The seedlings at approximately the V1 stage (first trifoliate unfolded) after the sub-zero temperature event were analyzed for survival post-frost as well as pre- and post-frost ureide content. Planting dates used for analysis ranged from August 18 to September 2 (Table C-2). Similarly, the first significant sub-zero temperature events ranged from September 12 to October 3. All data analyzed were from plants between 24 days and 34 days old (Table 3.1).

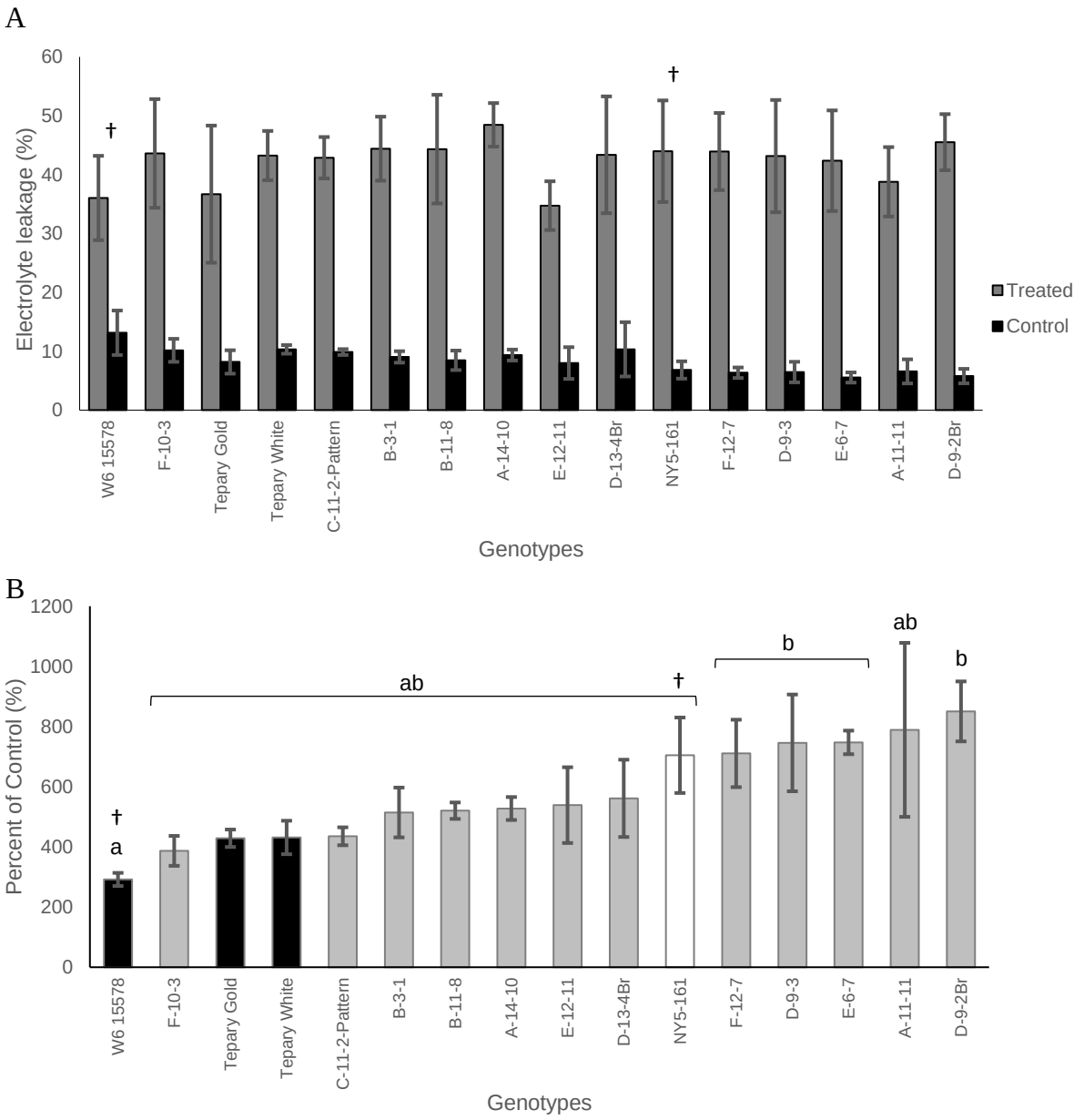
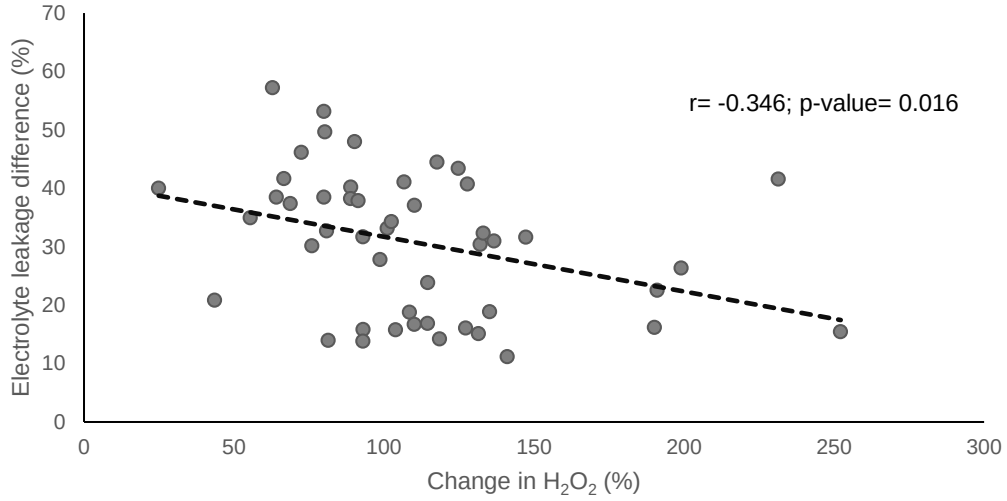


Figure 3.36: Cell death after a sub-zero temperature stress. Change in electrolyte leakage between the treated samples and control samples in 16 genotypes after treatment in a cold bath shown on a percent of control (A) and a difference from control (B) basis. Three tepary beans (tepary gold, tepary white and W6 15578), one common bean (NY5-161) and twelve interspecific introgression lines were analyzed. Bars indicate averages of 4-5 replicates ± standard error. Differences denoted by lower letters in (B) were determined by a post hoc Tukey's test. In (B), the tepary beans are shown in black, the common bean in white and the interspecifics in gray. Parents are indicated with a dagger symbol (†).

A



B

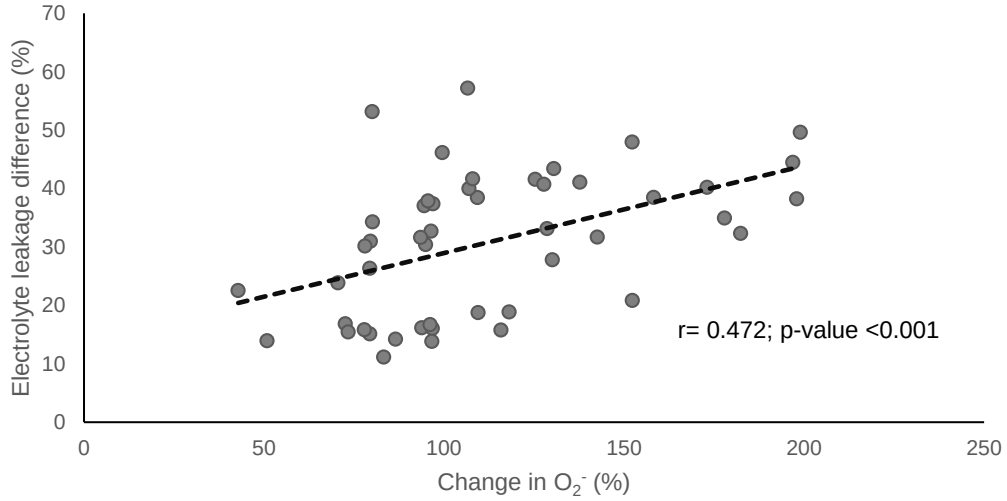


Figure 3.37: Correlations between ROS and cell death after sub-zero temperature stress. Leaf disks from fully expanded young leaves of nitrogen-fertilized common bean, tepary bean and interspecific introgression lines of common bean and tepary bean were subjected to sub-zero temperature stress. The difference in electrolyte leakage between the treated leaves and the control leaves correlated with the change in H_2O_2 (A) and O_2^- (B) of the same treated and control.

Table 3.1: Recorded sub-zero temperatures and duration during the field trials.

Year	Date Planted	Date of Sub-Zero Event	Days after sowing	Minimum Temp (°C)	Hours Below Zero
2013	August 30	October 3	34	-4.6	12.5
2014	August 18	September 12	24	-1.5	4
		September 14	27	-2.7	6
	September 2	October 3	31	-3.5	8
2015	August 26	September 22	27	-2.0	2.5
		September 28	33	-2.7	7

Minimum temperatures and duration of sub-zero temperature varied between plantings and likely had an effect on the data. The lowest initial sub-zero temperature event occurred in 2013 where the first damaging frost was -4.6°C. This event was also the longest in duration, with sub-zero temperatures lasting for 12.5 hours. The least severe frost event occurred during the first planting in 2014 where temperatures only reached -1.5°C. The shortest sub-zero temperature event occurred in 2015, when temperatures were only below freezing for 2.5 hours (Table 3.1). Two trials were analyzed in 2014 as the second trial had not fully emerged and avoided the first sub-zero temperature event.

Three tepary beans were removed from the analysis due to lack of germination or lack of available seed for the planting. Analysis on genotypes was completed on the remaining 137 genotypes and group analysis was completed on the 3 known groups: common beans, tepary beans and interspecific introgression lines. Levene's test revealed heterogeneous variance across years, therefore data were analyzed separately.

3.9.1. Survival of tepary bean, common bean and interspecific introgression lines one and seven days after a sub-zero temperature stress

The sub-zero temperature stress in 2013 killed almost the entire population (Figure 3.38 A) and there was no difference among survival of genotypes (Table A-24). There was a significant difference between groups, with tepary beans demonstrating a higher percentage of survival than the interspecific introgression lines. The mean survival of tepary beans and interspecific introgression lines was also greater than common bean (Table 3.2). No survival data after seven days was recorded for 2013 because plants surviving the frost were removed from the field and continued to be grown in a greenhouse to be used to provide seed for further experiments.

In the first 2014 frost trial >99% of plants were still alive one day post-frost, therefore data are only presented for survival 7 days after the frost. Survival differed between genotypes and between groups (Figure 3.39). Tepary beans had a higher percent survival than common beans and interspecific introgression lines (Table 3.2). Again, the interspecific introgression lines had a higher average survival than common beans, but the difference was not significant (Table A-24).

Survival one day and seven days after the sub-zero temperature stress trial was significantly different between genotypes in the second frost trial of 2014 (Table A-24). After one day, the

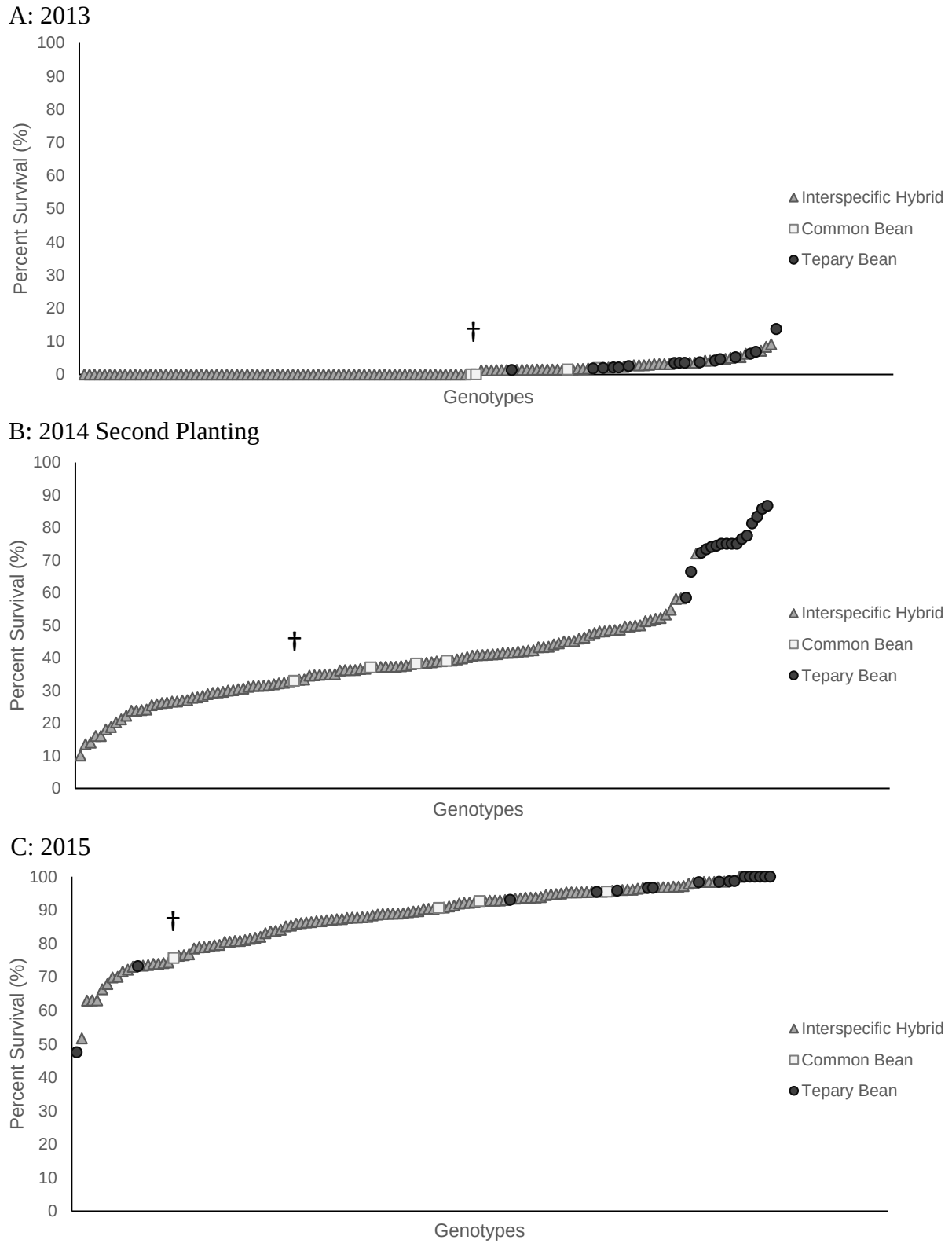


Figure 3.38: Percent survival of genotypes one day after the sub-zero temperature event. Comparison of average survival of genotypes one day after sub-zero temperature stress in 2013 (A), the second trial in 2014 (B) and 2015 (C). Plantings included four replicates. The first planting of 2014 showed little to no plant death and therefore data are not presented for this time point. The dagger symbol (†) denotes the common bean parent, NY5-161.

Table 3.2: Percent survival of genotypes after the sub-zero temperature stress.

One Day after Sub-Zero Temperature

	2013	2014 First Planting	2014 Second Planting	2015
	% Survival ± Standard Error			
Common Bean	0.6 ± 0.6 [ab]	N/A	36.1 ± 5.4 [a]	87.3 ± 6.3 [ab]
Interspecific Introgression Line	1.0 ± 0.2 [a]	N/A	36.9 ± 0.8 [a]	86.9 ± 0.8 [a]
Tepary Bean	3.7 ± 0.8 [b]	N/A	77.2 ± 2.7 [b]	95.6 ± 1.5 [b]

Seven Days after Sub-Zero Temperature

	2013	2014 First Planting	2014 Second Planting	2015
	% Survival ± Standard Error			
Common Bean	N/A	7.1 ± 2.4 [a]	25.0 ± 4.5 [a]	20.8 ± 3.0 [ab]
Interspecific Introgression Line	N/A	10.2 ± 0.6 [a]	22.5 ± 0.7 [a]	28.3 ± 0.9 [b]
Tepary Bean	N/A	60.6 ± 6.4 [b]	41.6 ± 4.0 [b]	35.3 ± 2.5 [a]

Values represent survival as a percentage of germination. Numbers in the same column with different letters are significantly different at $p<0.05$. N/A= data not available for this time point.

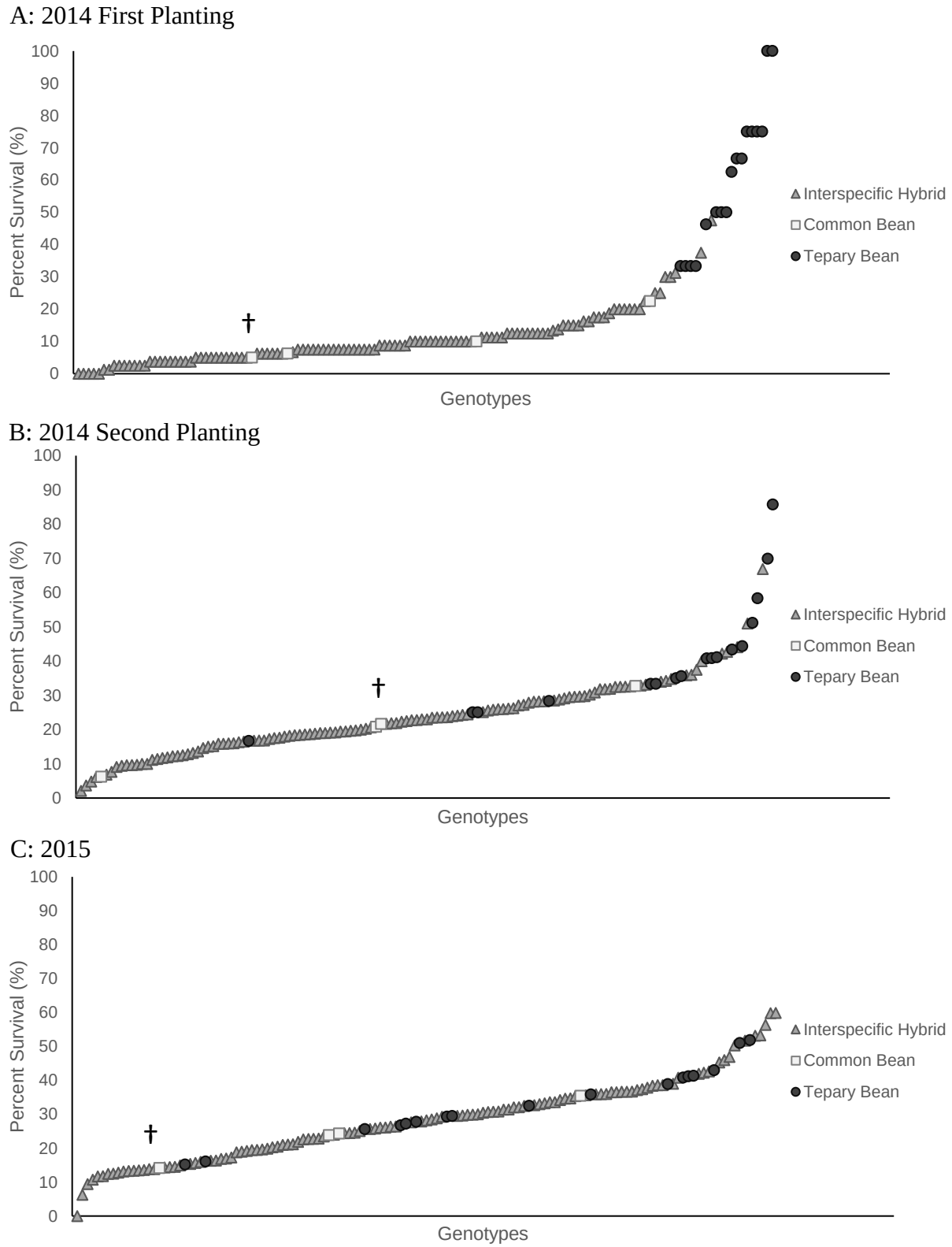


Figure 3.39: Percent survival of genotypes seven days after the sub-zero temperature event. Survival of genotypes seven days after sub-zero temperature stress in the first trial in 2014 (A), the second trial in 2014 (B), and 2015 (C). Plantings included four replicates. Surviving genotypes in 2013 were rescued from the field two days after the frost, so no 7 day data was recorded. The dagger symbol (†) denotes the common bean parent, NY5-161.

tepary beans had the highest percent survival compared to interspecific introgression lines and common beans (Table 3.2). After seven days tepary beans still had the greatest percent survival, however, unlike other trials, common beans had a greater average percentage survival than interspecific introgression lines (41.6%, 25.0%, and 22.5%, for tepary beans, common beans and interspecific introgression lines, respectively). At neither time point were common beans and interspecific introgression lines statistically different (Figures 3.38 and 3.39).

One day after the 2015 sub-zero temperature stress event survival between genotypes was not different, but was significantly different after seven days (Table A-24). Differences were significant between groups, both one day and seven days after the stress event. Tepary beans had the highest average survival, significantly different than the interspecific introgression lines but not the common beans at $p<0.05$ (Table 3.2). Survival of common beans and interspecific introgression lines were not statistically different in 2015 (Figures 3.38 and 3.39).

3.9.2. Ureide content differs between genotypes after sub-zero temperature stress

Ureide quantity was determined in a subset of the population in 2013 and the first planting in 2014. The analysis included 17 tepary beans, 3 common beans, 25 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). This selection included all of the tepary beans and common beans grown in the trial and the interspecific introgression lines were selected in random from each of the six independent backcrosses (discussed in section 2.2.1). In 2014, four tepary beans were removed from analysis due to lack of tissue to assay. Plants were analyzed mid-day on the day before the frost (determined by forecast), hours after the frost, and one day after the frost.

In 2013, the average uric acid content of the population decreased after the being subjected to a sub-zero temperature stress (Table 3.3). In 2014, however, average uric acid content of the population increased from 0.44 nmol/mg DW to 0.65 nmol/mg DW. Average uric acid was greater in tepary bean than interspecific introgression lines and common beans in both 2013 and 2014 (Table 3.3). Uric acid content in leaves of tepary bean, common bean and interspecific introgression lines differed significantly between genotypes at all three time-points for both years (Figures 3.40 and 3.41 A). No interaction between time point and genotype

Table 3.3: Ureide content in leaves during sub-zero temperature stress.

2013

	Uric Acid (nmol (mg DW)$^{-1}$)			Allantoin (nmol (mg DW)$^{-1}$)			Allantoate (nmol (mg DW)$^{-1}$)		
	Before	Day Of	After	Before	Day Of	After	Before	Day Of	After
Common Bean	0.19 ± 0.14^{Aa}	0.14 ± 0.02^{Aa}	0.15 ± 0.02^{Aa}	1.87 ± 0.54^{Aa}	1.38 ± 0.42^{Aab}	1.91 ± 0.64^{Aa}	9.03 ± 2.16^{Aa}	3.91 ± 0.56^{Bab}	2.74 ± 0.67^{Ba}
Inter-specific Line	0.20 ± 0.02^{Aa}	0.14 ± 0.01^{Ba}	0.16 ± 0.00^{ABa}	1.43 ± 0.21^{Aa}	1.86 ± 0.32^{Aa}	4.97 ± 0.42^{Bb}	7.40 ± 0.68^{Aa}	5.23 ± 0.54^{Ba}	6.70 ± 0.47^{ABb}
Tepary Bean	0.44 ± 0.03^{Ab}	0.28 ± 0.02^{Bb}	0.36 ± 0.04^{ABb}	2.12 ± 0.39^{Aa}	0.77 ± 0.13^{Bb}	2.09 ± 0.21^{Aa}	5.37 ± 0.82^{Aa}	1.48 ± 021^{Bb}	2.41 ± 0.24^{Ba}
Total Pop.	0.29 ± 0.02^{A}	0.19 ± 0.01^{B}	0.23 ± 0.02^{AB}	1.70 ± 0.19^{A}	1.45 ± 0.19^{A}	3.64 ± 0.27^{B}	6.75 ± 0.51^{A}	3.81 ± 0.72^{B}	4.79 ± 0.32^{B}

2014 (first population)

	Uric Acid (nmol (mg DW)$^{-1}$)			Allantoin (nmol (mg DW)$^{-1}$)			Allantoate (nmol (mg DW)$^{-1}$)		
	Before	Day Of	After	Before	Day Of	After	Before	Day Of	After
Common Bean	0.36 ± 0.04^{Aa}	0.40 ± 0.03^{Aa}	0.53 ± 0.05^{Bab}	0.58 ± 0.26^{Aa}	1.20 ± 0.19^{Aa}	5.04 ± 1.36^{Ba}	5.35 ± 1.41^{Aa}	5.80 ± 0.64^{Aa}	17.78 ± 3.85^{Ba}
Inter-specific Line	0.37 ± 0.01^{Aa}	0.45 ± 0.01^{Ba}	0.42 ± 0.02^{Aa}	1.51 ± 0.19^{Aa}	1.79 ± 0.40^{Aa}	2.57 ± 0.26^{Aa}	6.90 ± 0.55^{Aa}	9.54 ± 1.28^{Aa}	9.90 ± 0.77^{Aa}
Tepary Bean	0.56 ± 0.03^{Ab}	0.67 ± 0.03^{Ab}	1.13 ± 0.40^{Ab}	2.54 ± 0.68^{Aa}	2.76 ± 0.71^{Aa}	4.32 ± 0.93^{Aa}	6.28 ± 1.42^{Aa}	9.43 ± 1.80^{ABa}	13.84 ± 2.32^{Ba}
Total Pop.	0.44 ± 0.01^{A}	0.50 ± 0.01^{A}	0.65 ± 0.13^{A}	1.77 ± 0.24^{A}	1.97 ± 0.32^{A}	3.25 ± 0.35^{B}	6.53 ± 0.55^{A}	9.17 ± 0.95^{AB}	11.56 ± 0.93^{B}

Upper case letters signify differences within the row for each individual ureide and lower case letters indicate differences in the columns (between species) when values were significantly different at $p<0.05$.

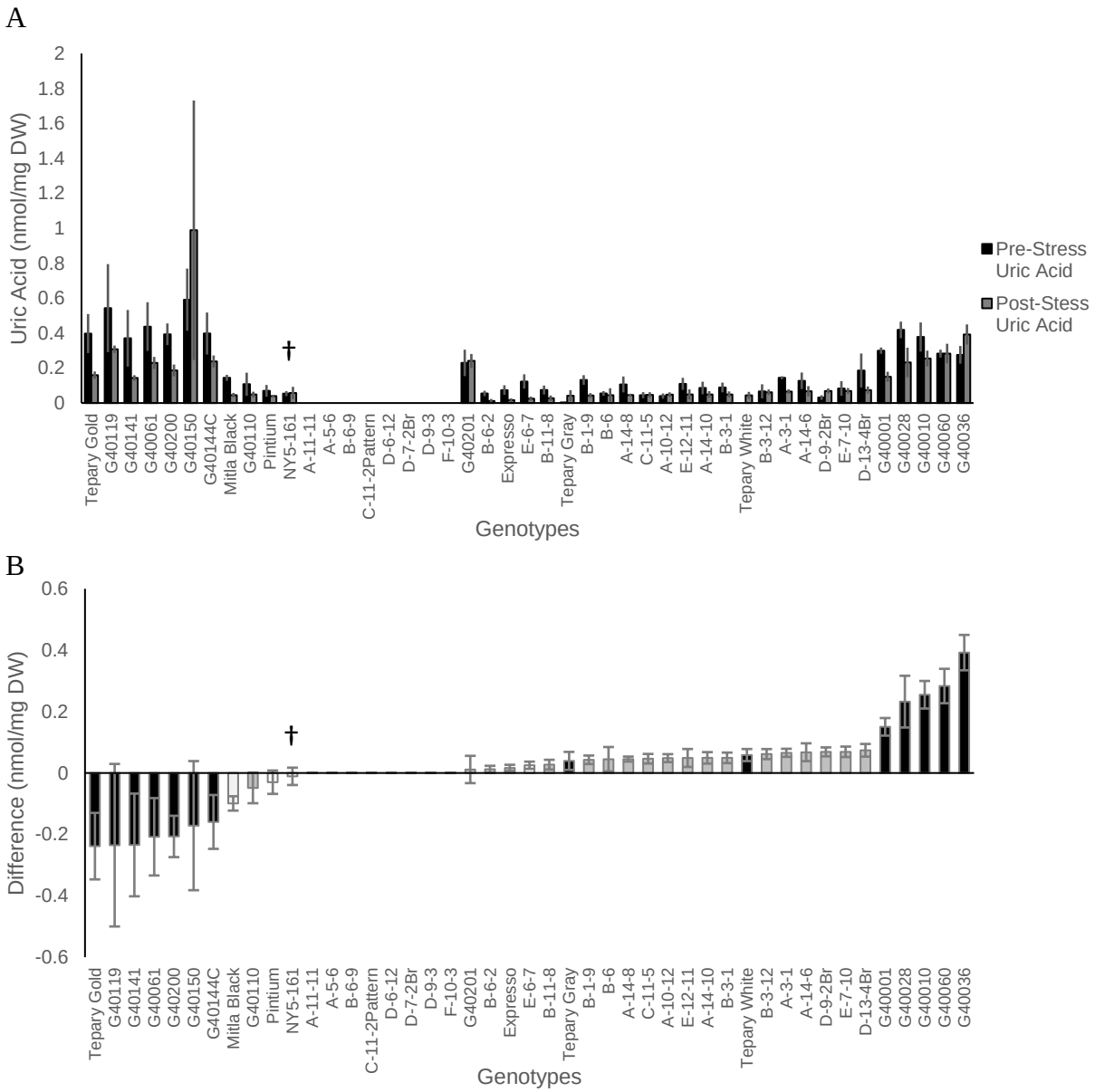


Figure 3.40: Uric acid content in a sub-zero temperature trial in 2013. Uric acid content in leaf tissue before and two days after a sub-zero temperature stress in 2013 (A) and the difference (B) between the two quantities reported in (A). Uric acid was quantified in 17 tepary beans, 3 common beans, 25 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of uric acid (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger symbol (†).

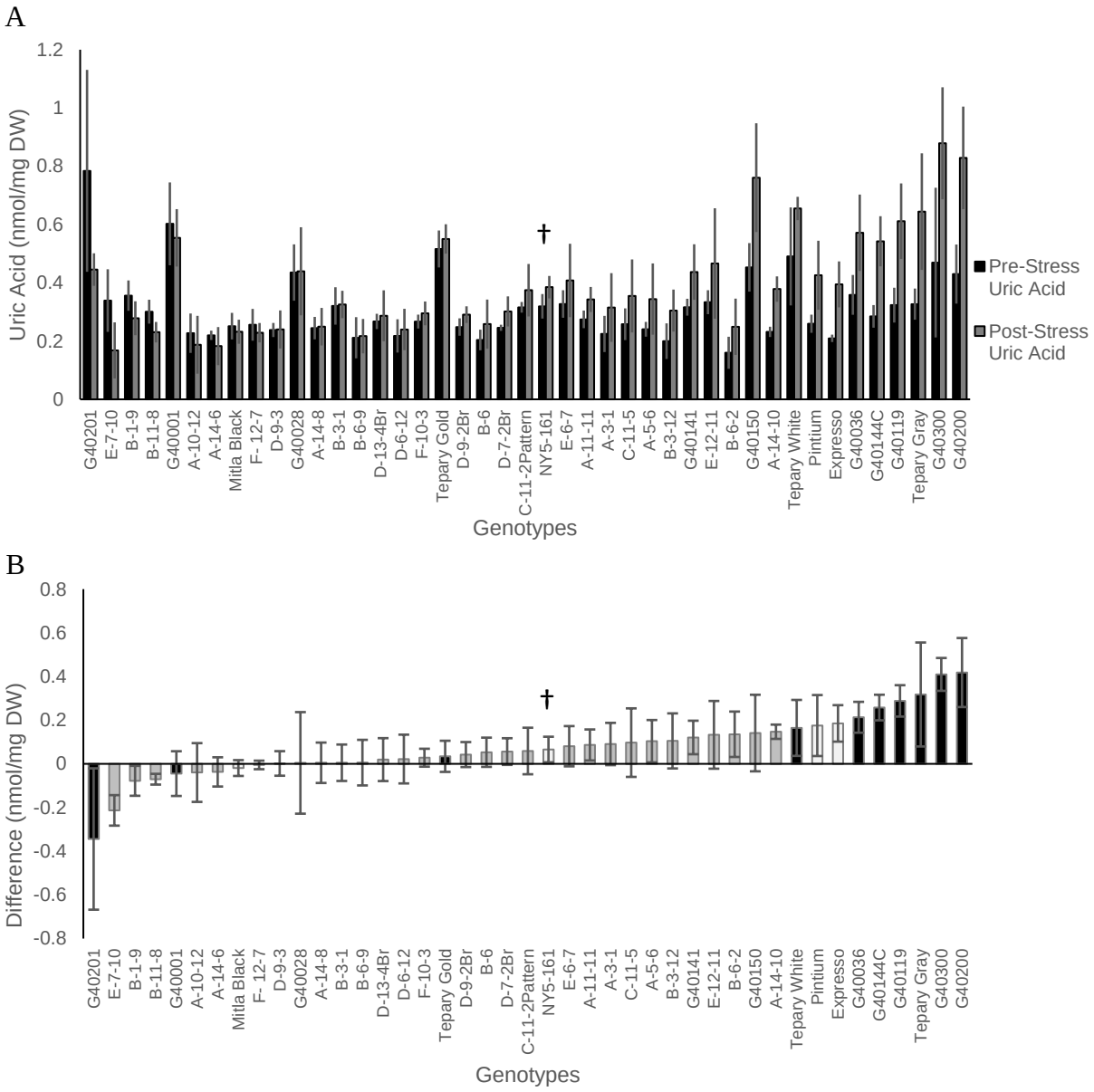


Figure 3.41: Uric acid content in a sub-zero temperature trial in 2014. Uric acid content in leaf tissue before and two days after a sub-zero temperature stress in 2014 (A) and the difference (B) between the two quantities reported in (A). Uric acid was quantified in 13 tepary beans, 3 common beans, 26 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of uric acid (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger (†).

occurred in either year, determined by a two-way ANOVA (Table A-25). The differences between amounts of uric acid after the stress and the amount of uric acid before the stress was calculated and the effect of genotype was not significant for either 2013 or 2014 (Figures 3.40 and 3.41 B).

The average allantoin content of the population increased in both 2013 (1.70 nmol/mg DW to 3.64 nmol/mg DW) and 2014 (1.77 nmol/mg DW to 3.25 nmol/mg DW) after the stress. Before the 2013 sub-zero temperature stress, similar average allantoin was analyzed in tepary beans, common beans and interspecific introgression lines, however after the interspecific introgression lines contained more allantoin than the common beans and the tepary beans (Table 3.3). No difference in allantoin content was significant in 2014 between tepary beans, common beans and interspecific introgression lines. On an individual genotype basis, both genotype and cold stress had significant effects on allantoin quantity in 2013 and 2014 (Figures 3.42 and 3.43 A). There was a significant interaction between day and genotype for 2013, but not for 2014 (Table A-25). The difference of allantoin after the stress to before the stress was calculated and genotypes differed in 2013 but not 2014 (Figures 3.42 and 3.43 B).

Average allantoate of the population decreased due to stress in 2013 but increased in 2014 (from 6.53 nmol/mg DW to 11.56 nmol/mg DW). Similar to the relationship between groups with allantoin content, more allantoin was measured in the interspecific introgression lines after the stress than in the tepary and common beans in 2013, but no differences were significant in 2014 (Table 3.3). Differences in allantoate quantity existed between genotypes and between the sampling times in 2013 and 2014 (Figures 3.44 and 3.45 A). A significant interaction between time point and genotype also occurred in both years (Table A-25). Genotypic differences existed in the variability of allantoate quantity after to before the stress in 2013 but not 2014, determined by a one-way ANOVA (Figures 3.44 and 3.45 B). Values for ureide content of individual genotypes can be found in Table D-1 and D-2.

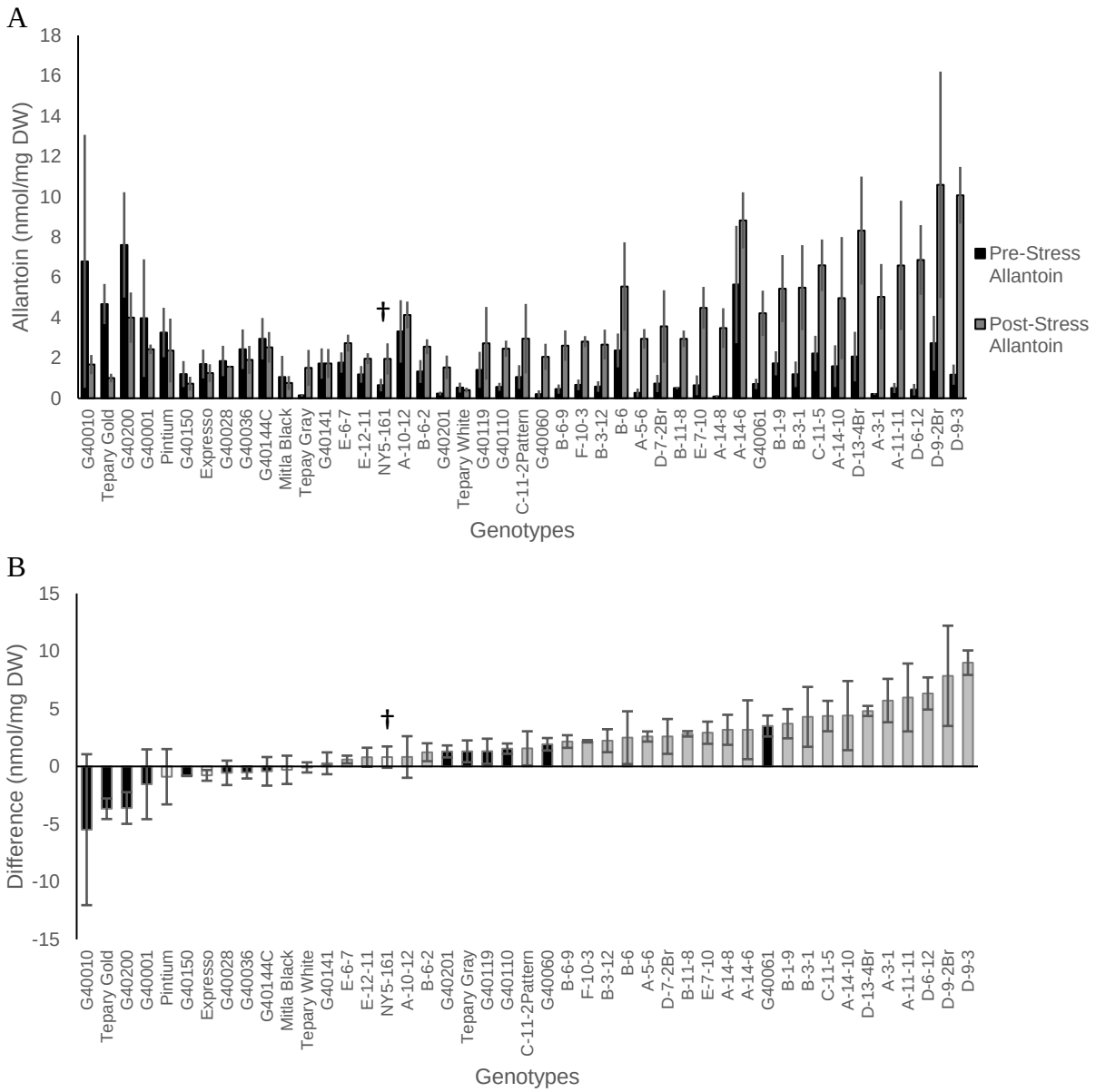


Figure 3.42: Allantoin content in a sub-zero temperature trial in 2013. Allantoin content in leaf tissue before and two days after a sub-zero temperature stress in 2013 (A) and the difference (B) between the two quantities reported in (A). Allantoin was quantified in 17 tepary beans, 3 common beans, 25 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of allantoin (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger (†).

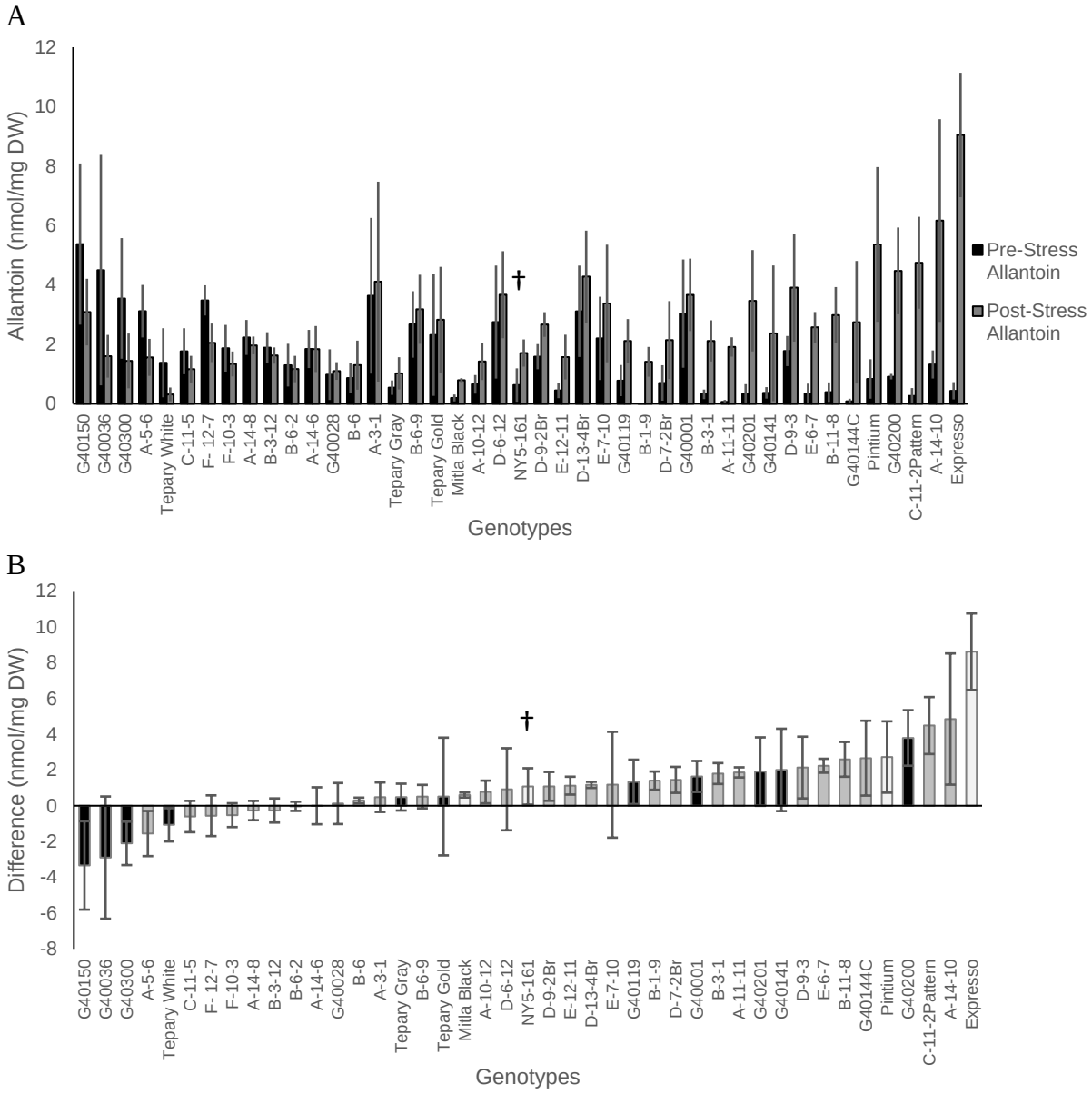


Figure 3.43: Allantoin content in a sub-zero temperature trial in 2014. Allantoin content in leaf tissue before and two days after a sub-zero temperature stress in 2014 (A) and the difference (B) between the two quantities reported in (A). Allantoin was quantified in 13 tepary beans, 3 common beans, 26 interspecific introgression lines, and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of allantoin (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger (†).

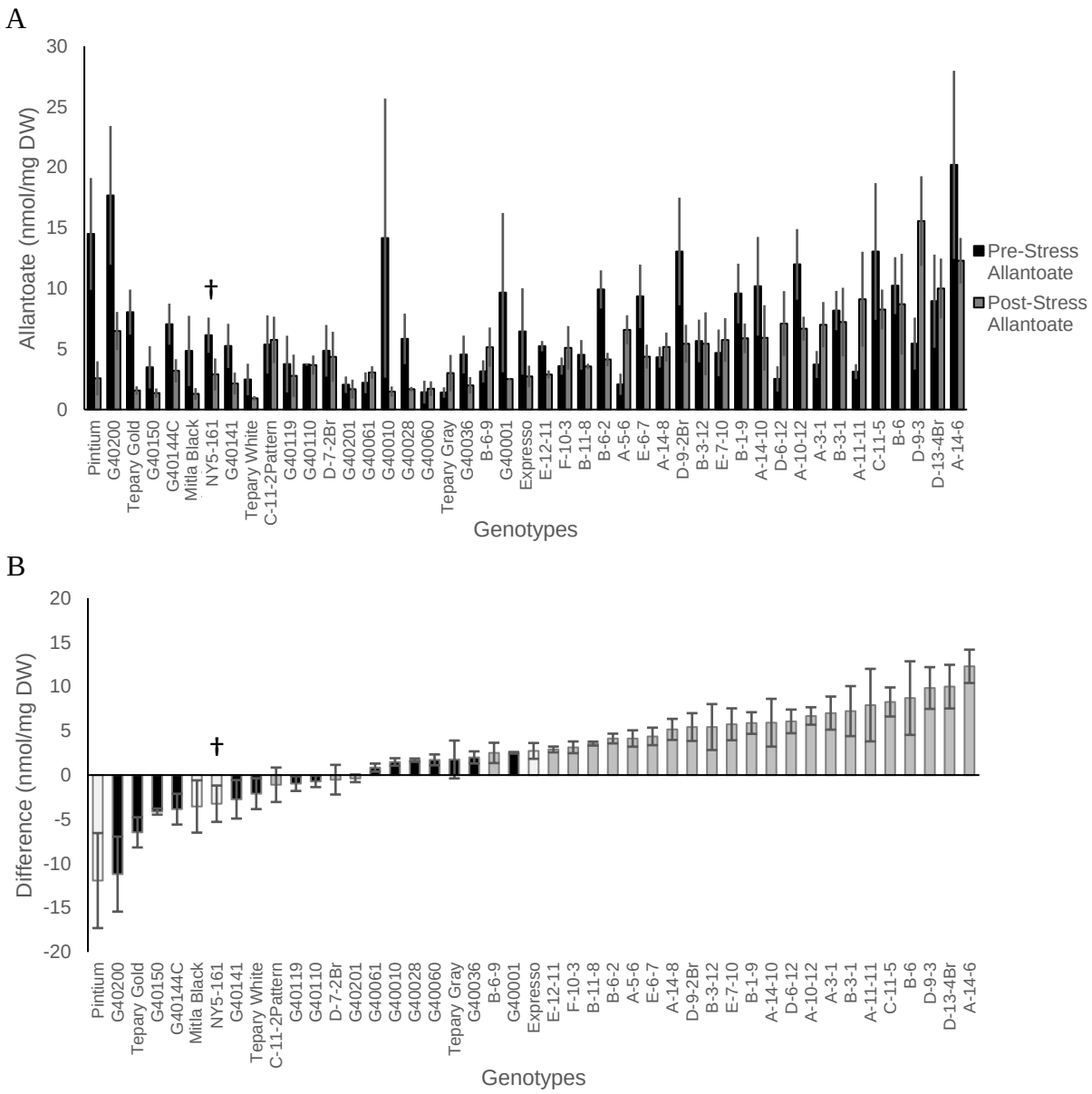


Figure 3.44: Allantoate content in a sub-zero temperature trial in 2013. Allantoate content in leaf tissue before and two days after a sub-zero temperature stress in 2013 (A) and the difference (B) between the two quantities reported in (A). Allantoate was quantified in 17 tepary beans, 3 common beans, 25 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of allantoate (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger (†).

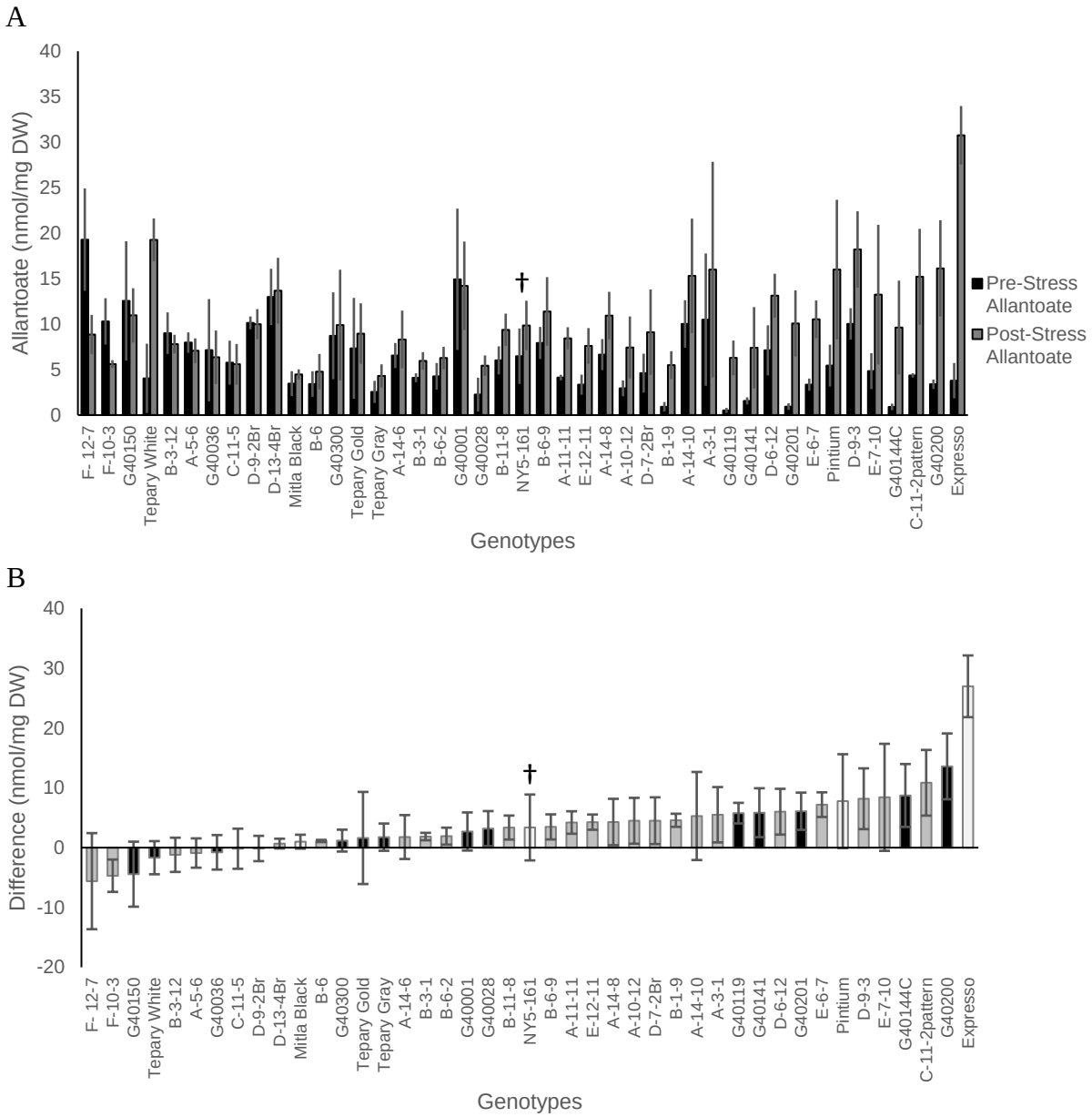


Figure 3.45: Allantoate content in a sub-zero temperature trial in 2014. Allantoate content in leaf tissue before and two days after a sub-zero temperature stress in 2014 (A) and the difference (B) between the two quantities reported in (A). Allantoate was quantified in 13 tepary beans, 3 common beans, 26 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Bars show the average of 3 or 4 replicates ± standard error. The quantity of allantoate (A) is ordered based on the difference between post- and pre-stress (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray. NY5-161 is denoted by the dagger (†).

3.9.3. Correlations between ureide quantity and agronomic traits after sub-zero temperature stress

The amount of uric acid, allantoin, and allantoate from the three sampling time points (one day before the stress, hours after the stress, and one day after the stress) was analyzed for correlation with the percentage of plants that survived the stress (one day post stress in 2013 and seven days post stress in 2014) (Table A-27). In 2013, the amount of uric acid after the stress was weakly correlated with percent of plants alive (r=0.180). Since much of the planting died, it was possible correlations were muted by not having enough variability in the population (Table A-26). In 2014, percentage of plant survival correlated with the quantity of uric acid before the stress (r=0.390), uric acid during the stress (r=0.447), uric acid after stress (r=0.198) and the difference between uric acid between before and after the stress (r=0.168) (Table A-27) (Figure 3.46). As no explanation could be made for the uric acid outlier, the data was included in the analysis.

Not surprisingly, specific ureide concentrations were highly correlated with each other. Allantoin and allantoate were tightly correlated at the same time points in both 2013 and 2014 (r between 0.746 and 0.961 for all combinations) (Table A-26 and A-27). Uric acid content before, during and after the stress was negatively correlated with allantoate during and after the stress and allantoin after the stress in 2013. However, in 2014, uric acid was positively correlated with allantoin and allantoate within, but not between, sampling times. The content of a specific ureide at one time point was usually correlated with quantity of the same ureide at the other time points (see Table A-26 and A-27 for full correlation analysis and Figure D-1 for graphs).

The average percent survival was also compared with the average change in ROS and cell death from the controlled sub-zero temperature experiment (Section 3.8). Neither the percent cell death nor the difference in cell death statistically correlated with the average field survival in the first planting of 2014, the second planting of 2014, or 2015. The change in O_2^- also did not statistically correlate to the field results. The change in H_2O_2 immediately after the treatment, however, correlated to the survival of both the first and the second plantings in 2014 (r=0.672 and r=0.585, respectively) and the change in H_2O_2 24 hours after the treatment correlated to the average survival in the first planting in 2014 (r= 0.568) (Figure 3.46).

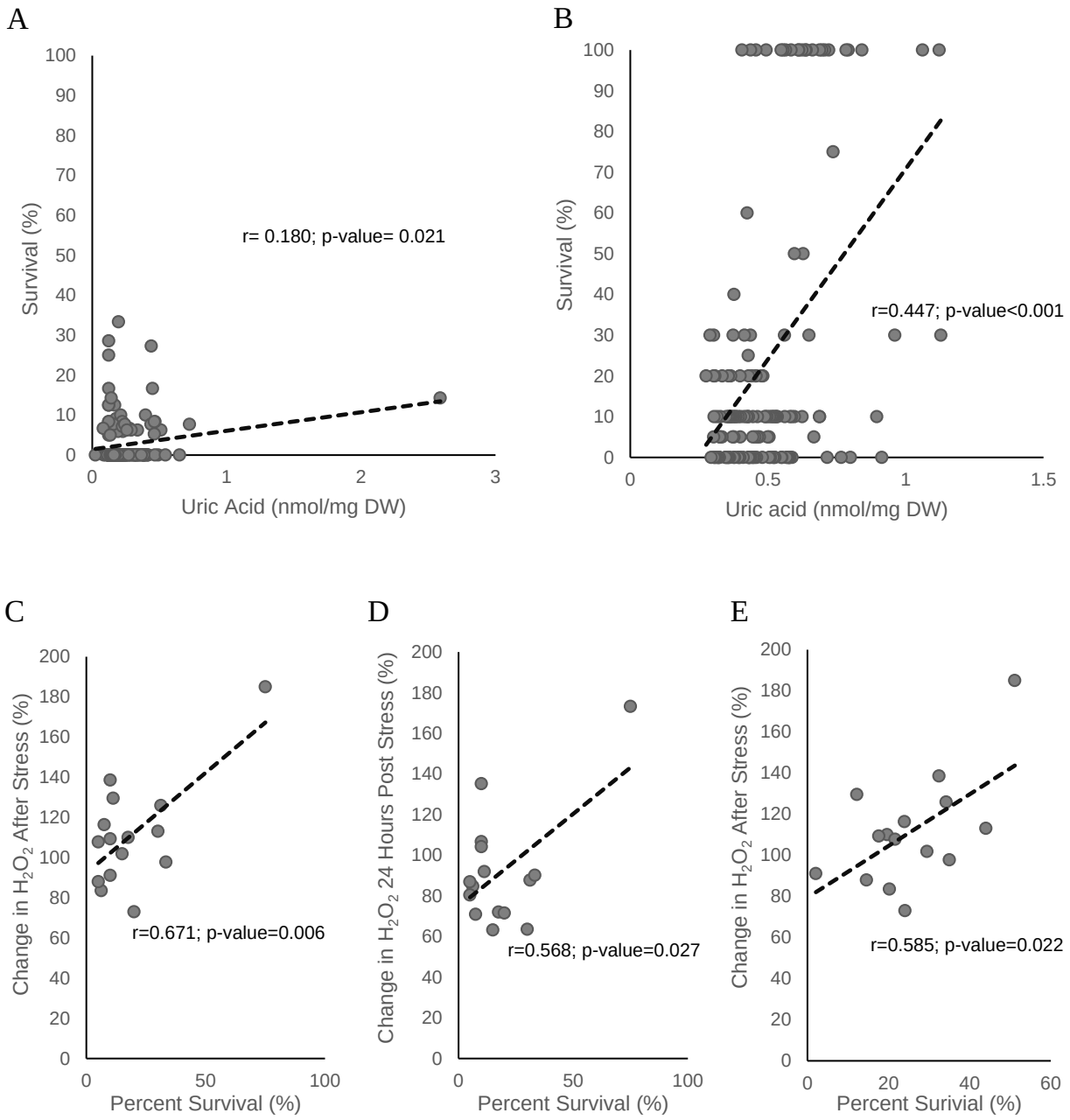


Figure 3.46: Sub-zero temperature trial correlations. Uric acid measurements were taken before, during and after a sub-zero temperature event in the field. Significant correlations were demonstrated with plant survival in 2013 (A) and 2014 (B). The correlation between uric acid after the stress (A) and during stress (B) and survival is depicted. Other correlations can be found in Table A-26 and A-27. Correlation analysis was also completed between average survival in the field in the first planting of 2014 (C and D) and the second planting of 2014 (E) and the average change in H_2O_2 in the tissue in the controlled sub-zero temperature stress experiment immediately after the treatment (C and E) and 24 hours after the treatment (D).

3.10. Water limitation field experiment

In 2013, four replicates of 117 interspecific introgression lines, 3 common beans, 4 tepary beans, and 1 bean of unknown species designation (Mitla Black) were planted in the USDA-ARS in Isabela, PR in a split plot design with group balanced blocks (Table 3.4). At the beginning of flowering, irrigation was discontinued to two replicates. A Drought Intensity Index (DII; 1-average $yield_{stressed}$/average $yield_{non\text{-}stressed}$) determined the stress in 2013 was moderate (DII=0.40 or 0.42 if tepary beans are removed from the calculation). Ureide content of leaves was measured 4, 6, 8, and 10 days after water withdrawal. Yield, other agronomic traits (discussed in Appendix E), and drought scores were documented to determine differences in stress response between genotypes and between interspecific introgression lines, common beans and tepary beans.

In 2014, three treatment and three control replicates of 89 interspecific introgression lines, 3 common beans, 4 tepary beans and 1 bean of unknown species designation (Mitla Black) were planted in the USDA ARS in Isabela, PR (Table 3.4). The DII in 2014 determined the drought stress was mild (DII=0.22 or 0.24 if tepary beans are removed from the calculation). Yield and drought scores were analyzed and compared with ureide accumulation 12 days after water was removed. Earlier samples were not analyzed as rainfalls occurred early on the trial and symptoms did not show up until later in the trial. Other agronomic traits are discussed in Appendix E.

Yield data and drought scores were also collected in 2015 although a decision was made not to collect tissue for ureide analysis based on results of previous years. Four treatment and four stress replicates of 86 interspecific introgression lines, 3 common beans, 4 tepary beans and 1 bean of unknown species designation (Mitla Black) were sown. Irrigation was discontinued at flowering to the treatment replicates (Table 3.4). The DII in 2015 determined the drought stress was mild (DII=0.26 or 0.28 if tepary beans are removed from the calculation).

3.10.1. Agronomic measurements of tepary bean, common bean and interspecific introgression lines under water limitation stress

In 2013, yield differed among genotypes, between stressed and unstressed treatment and there was an interaction between genotypes and stress (Table A-28). When genotypes were categorized as tepary beans, common bean or hybrids, the effect of group, stress and the interaction between the group and the stress were all significant. The average yield of the interspecific

Table 3.4: Summary of Puerto Rico water limitation field trials.

Year	Planting Date	Removal of Irrigation Date	Plant Stage	Drought Intensity Index (1-average $yield_{Stress}$/average $yield_{Control}$)
2013	January 17	February 22	Beginning of Flowering	0.42 (moderate)
2014	January 17	February 11	Beginning of Flowering	0.24 (mild)
2015	January 28	February 23	Beginning of Flowering	0.28 (mild)

Table 3.5: Yield in water limitation stress trial.

	2013		2014		2015	
	Limited (kg/ha)	Control (kg/ha)	Limited (kg/ha)	Control (kg/ha)	Limited (kg/ha)	Control (kg/ha)
Common Bean	827.0 ± 54.8^{Aa}	1244 ± 73.5^{Ba}	1258.5 ± 137.8^{Aa}	1330.2 ± 177.4^{Ba}	845.9 ± 86.9^{Aa}	1026.2 ± 95.0^{Ba}
Inter-specific Line	493.16 ± 14.4^{Ab}	857.3 ± 20.7^{Bb}	676.2 ± 15.9^{Ab}	920.7 ± 24.2^{Bb}	484.0 ± 11.2^{Ab}	678.7 ± 19.9^{Bb}
Tepary Bean	1093.5 ± 100.6^{Aa}	846.75 ± 100.2^{Bab}	1904.9 ± 259.5^{Ac}	2575.5 ± 414.0^{Bc}	1414.7 ± 72.5^{Ac}	1579.8 ± 72.4^{Bc}
Total Pop.	523.0 ± 15.7^{A}	869.9 ± 19.9^{B}	748.9 ± 24.1^{A}	995.1 ± 32.6^{B}	546.6 ± 16.0^{A}	742.6 ± 22.4^{B}

Upper case letters signify differences between water-limited and control (for the individual year) and lower case letters indicate differences in the columns at $p < 0.05$.

introgression lines decreased (857 kg/ha to 493 kg/ha) similar to the average yield of the common beans (1244 kg/ha to 827 kg/ha), however the average yield of the tepary beans increased under conditions of water limitation (847 kg/ha to 1094 kg/ha) (Table 3.5).

The yield of most genotypes decreased when irrigation was removed in 2013 (Figure 3.47 A,B). Individually, the average yield of the four tepary beans (Gray, White, Gold and G40001) and three interspecific introgression lines (D-5-10, D-1-5 and C-11-2Pattern) increased under the water limitation. Most interspecific introgression lines yielded lower than the common beans under irrigated and stress conditions, but a few had comparable yield. The tepary beans had the highest yield under stress conditions and Tepary Gray yielded greater than NY5-161 and similar to Pintium under irrigated conditions (Figure 3.47 A,B).

Each line under the water-limited conditions was visually scored based on drought symptom of the leaves, with a score of 1 indicating no symptoms and 5 indicating all plants showing leaf curling and wilting. Average drought scores ranged between 1 and 5 with differences being significant for day 6, day 8 and day 10. Differences among groups were also significant (Table A-30), with tepary beans having lower scores than both common beans and interspecific introgression lines on day 6, day 8 and day 10 (Figure 3.47 C). The scores were not different between the common beans and the interspecific introgression lines.

In 2014, yield in a section of the irrigated block was reduced due to poor germination, therefore yield was first corrected by percent germination, then analyzed. Even though the drought stress was mild in 2014, it had a significant effect on yield. Genotypic differences and an interaction between genotype and treatment were also significant (Table A-33). The difference between common beans, tepary beans and interspecific introgression lines was also significant, with tepary beans having the highest yield and the interspecific introgression lines having the lowest yield regardless of water status (Table 3.5). Lower average yield was observed due to water limitation in all three groups, however the yield of some genotypes increased under water limitation (Figure 3.48 A,B).

Drought scores were taken by Dr. Tim Porch in the later stages of the drought treatment because the symptoms were not obvious during the ureide analysis period. Scores were on a scale of 1-8, with 1 being no symptoms and 8 being severely stressed. Drought scores were different among genotypes (Table A-35). Tepary beans showed fewer drought symptoms than common beans and interspecific introgression lines. The interspecific introgression lines, as a whole,

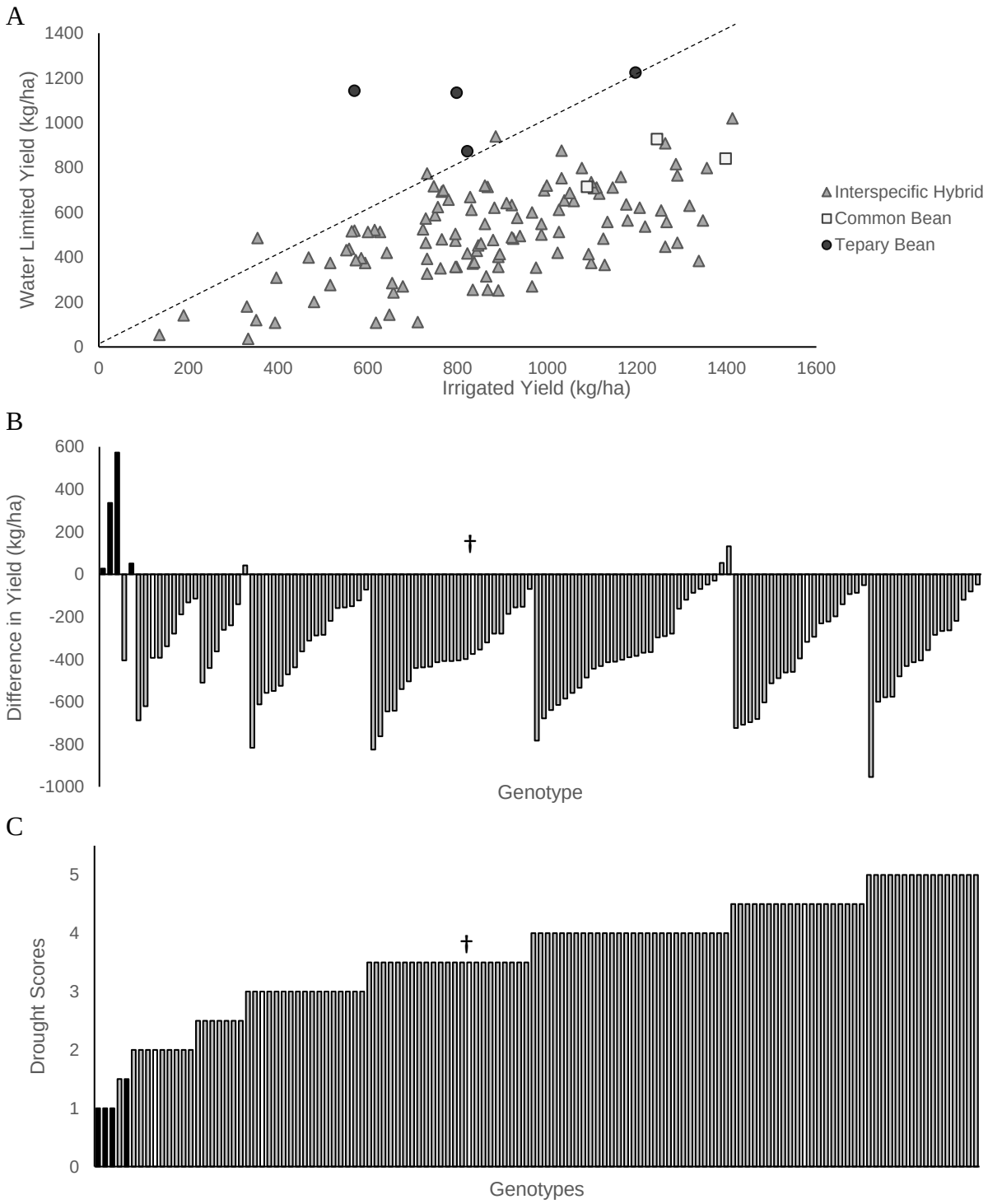


Figure 3.47: Yield and drought scores of the water limitation trial in 2013. Comparison of yield of genotypes during irrigation (population average 873.39 kg/ha) and with water removed at the beginning of flowering (population average 525.44 kg/ha) (A). Difference between the average water-limited yield and the average irrigated yield for individual genotypes (B). Drought scores on a 1-5 scale with 1 indicating no symptoms and 5 indicating worst leaf symptoms (C). Plants were scored six, eight, and ten days after irrigation was discontinued (shown is day 10). All values are the average of two replicates. The dotted line in (A) represents equal yield of the two conditions. The tepary beans are shown in black, the common beans in white and the interspecifics in gray. The order of genotypes in (B) and (C) is the same. The dagger symbol (†) denotes the common bean parent, NY5-161.

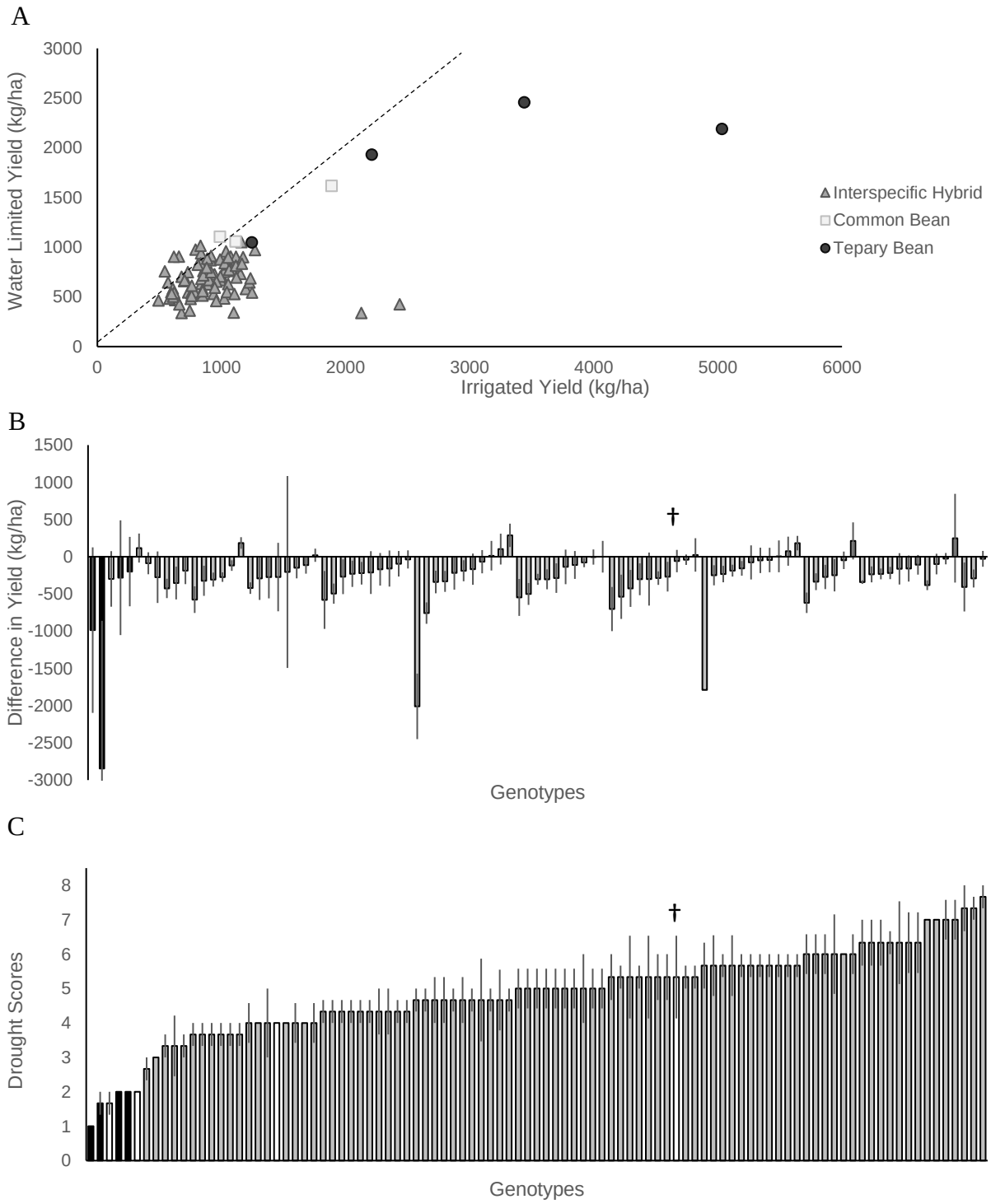


Figure 3.48: Yield and drought scores of the water limitation trial in 2014. Comparison of yield of genotypes during irrigation and with water removed at the beginning of flowering (A). Difference between the average water-limited yield and the average irrigated yield for individual genotypes (B). Drought scores on a 1-8 scale with 1 indicating no symptoms and 8 indicating worst leaf symptoms (C). All values are the average of three replicates ± propagation of standard error (B) or standard error (C). The dotted line in (A) represents equal yield of the two conditions. The tepary beans are shown in black, the common beans in white and the interspecifics in gray. The order of genotypes in (B) and (C) is the same. The dagger symbol (†) denotes the common bean parent, NY5-161.

showed greater drought symptoms than the common beans, however several lines performed better than the common bean parent (Figure 3.48 C).

The yield in 2015 was significantly affected by genotype and by treatment. No interaction occurred between the genotype and the treatment (Table A-38). The yield of all three groups were significantly different, with tepary having the overall highest yield and the interspecific introgression lines having the average lowest yield (Figure 3.49 A,B). The average yield of interspecific introgression lines (679 kg/ha to 484 kg/ha), common beans (1026.21 kg/ha to 845 kg/ha), and tepary beans (1580 kg/ha to 1415kg/ha) was lower in the water-limited blocks (Table 3.5).

Drought scores were different among genotypes and among groups (Table A-38). On average, tepary beans had the lowest drought scores and the interspecific introgression lines had the highest drought scores (Figure 3.49 C).

3.10.2. Ureide content in leaves after water limitation stress

In 2013, leaves of a subset of the population were collected 4, 6, 8, and 10 days after stress to analyze the ureide content changes due to the stress. This subset contained 15 interspecific introgression lines, with genotypes from each of the independent back crosses were represented in the analysis (see section 2.2.1), and the common bean parent, NY5-161. A Repeated Measure ANOVA indicated that no differences existed due to day, day*genotype, day*treatment, and day*genotype*treatment in uric acid quantity, although individual two-way ANOVAs on each day determined significance in the interaction of genotype and stress on day 6 and genotype, treatment and the interaction of genotype and treatment on day 8. For allantoin content, the day, day*genotype, day*treatment, and day*genotype*treatment interactions were all significant (after correction by Greenhouse-Geisser Epsilon). Day and day*treatment were significant in allantoate content (after correction by Greenhouse-Geisser Epsilon) (Figure D-3; Table A-29).

In 2014, ureide analysis was completed on leaf tissue of a larger subset of the population to determine if ureide levels could be correlated to drought stress. The subset included three common beans, four tepary beans, 37 interspecific introgression lines of tepary bean and common bean and one bean of unknown species designation (Mitla Black). As rainfall occurred at the beginning of the trial, only tissue from 12 days after irrigation was discontinued was analyzed.

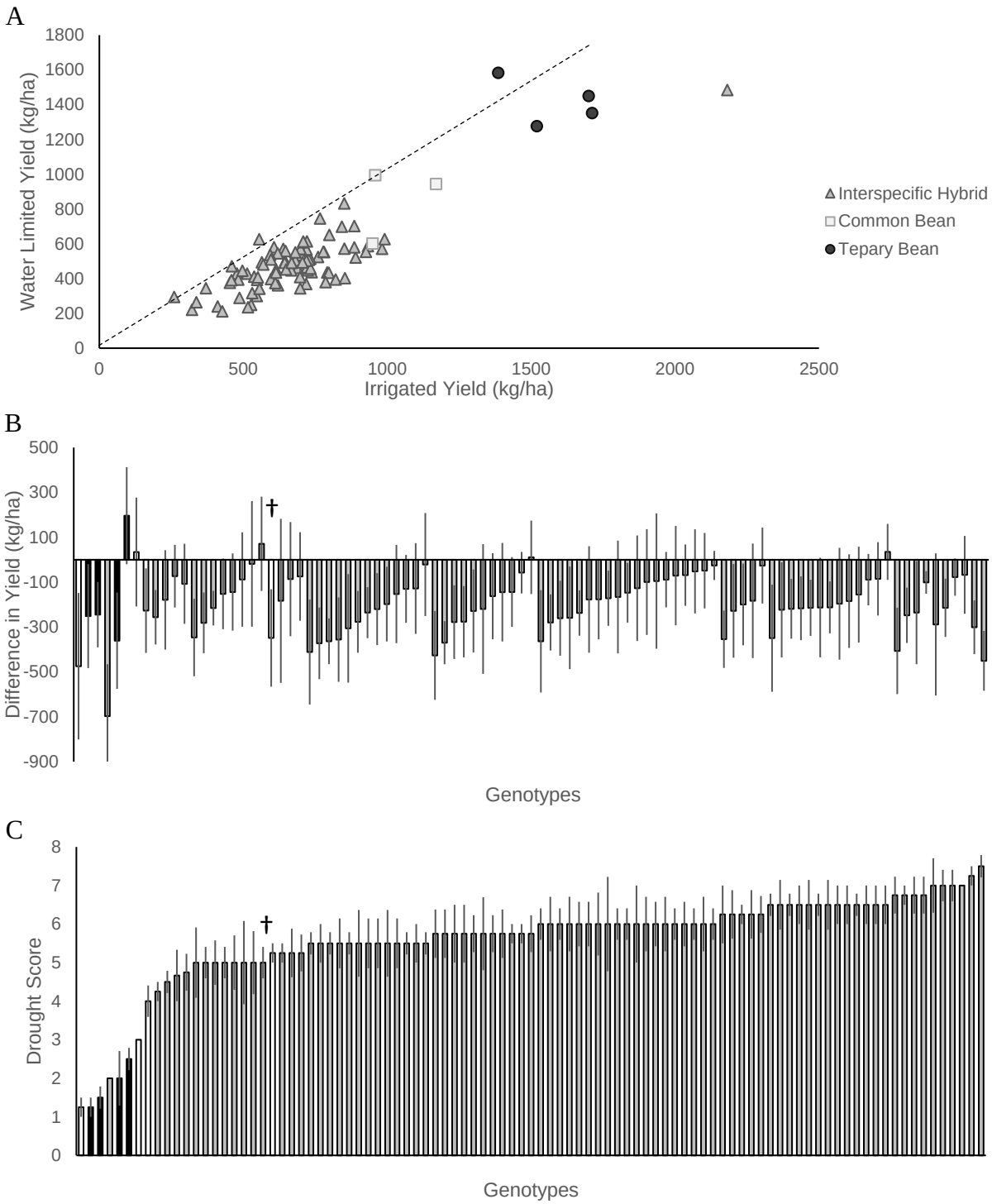


Figure 3.49: Yield and drought scores of the water limitation trial in 2015. Four irrigated and four water-limited replications of 86 interspecifics, 3 common beans, 4 tepary beans and 1 bean of unknown species designation were planted in Isabela, PR in 2015. A comparison of the yield (A) and the difference between the stressed treatment and the control treatment yield (B) were calculated. Also shown are drought scores (C) on a 1-8 scale with 1 indicating no symptoms and 8 indicating worst leaf symptoms. All values are the average of four replicates ± propagation of standard error (B) or standard error (C). The dotted line in (A) represents equal yield of the two conditions. The tepary beans are shown in black, the common beans in white and the interspecifics in gray. The order of genotypes in (B) and (C) is the same. The dagger symbol (†) denotes the common bean parent, NY5-161.

The average uric acid content of the population was 0.2 nmol/mg DW and this level did not differ between the treated leaves and the control leaves. In the control samples, tepary beans contained less uric acid than the interspecific introgression lines, but was not different than the common beans (Table 3.6). There was no difference in uric acid content between the groups in the water-limited samples (Figure 3.50 A; Table A-34). Uric acid content varied between genotypes, but was not different due to the treatment and no interaction of genotype and stress was calculated. The general response of uric acid to the stress was mixed. The uric acid content of some genotypes increased due to the stress and decreased due to stress in others (Figure 3.50 B). This mixed response was not due to group as the tepary beans, the common beans and the interspecific introgression lines all had genotypes that increased and genotypes that decreased in uric acid content.

The average allantoin content of the population was greater in the leaves of the water-limited plants than the control plants. Similar to uric acid, tepary beans contained less allantoin than the interspecific introgression lines but levels were not different than common beans (Table 3.6). This relationship was consistent in the treated samples and the control samples. Allantoin content in interspecific introgression lines and common bean was also similar. On a specific genotype basis, allantoin content varied. Most genotypes contained increased allantoin in the water-limited samples compared to the control samples, however in some genotypes allantoin decreased following drought stress (Figure 3.51 A). Although this discrepancy was observed, the interaction between the genotype and the treatment was not significant (Table A-34). The tepary bean allantoin content seemed to be more stable than that of the interspecific introgression lines and common beans. Although allantoin content did change in response to water limitation in tepary bean, the changes in some of the genotypes in the other two groups were, in some cases, over twice as large (Figure 3.51 B).

Allantoate had the largest change due to water limitation, increasing from an average of 3.84 nmol/mg DW to 6.51 nmol/mg DW. The interspecific introgression lines contained more allantoate than tepary beans and common beans in the treated samples and more allantoate than the tepary beans in the control samples (Table 3.6). Allantoate content differed between genotypes and between the water-limited and control treatment. The interaction between the genotype and the treatment was not significant (Table A-34). Similar to allantoin, most of the genotypes analyzed had more allantoate in the leaves of the treated plants than the control plants, however

Table 3.6: Ureide content in leaves during water limitation stress in 2014.

	Uric Acid (nmol (g DW)$^{-1}$)		Allantoin (nmol (g DW)$^{-1}$)		Allantoate (nmol (g DW)$^{-1}$)	
	Limited	Control	Limited	Control	Limited	Control
Common Bean	0.21 ± 0.04^{Aa}	0.21 ± 0.03^{Aab}	0.61 ± 0.38^{Aab}	0.41 ± 0.18^{Bab}	3.55 ± 1.73^{Ab}	2.60 ± 1.19^{Aab}
Inter-specific Line	0.22 ± 0.01^{Aa}	0.24 ± 0.01^{Ba}	1.11 ± 0.09^{Aa}	0.64 ± 0.06^{Ba}	7.32 ± 0.41^{Aa}	4.26 ± 0.30^{Ba}
Tepary Bean	0.17 ± 0.01^{Aa}	0.16 ± 0.01^{Bb}	0.31 ± 0.07^{Ab}	0.14 ± 0.04^{Bb}	2.39 ± 0.34^{Ab}	1.58 ± 0.14^{Bb}
Total Population	0.21 ± 0.01^{A}	0.23 ± 0.01^{B}	0.98 ± 0.08^{A}	0.57 ± 0.05^{B}	6.51 ± 0.38^{A}	3.84 ± 0.27^{B}

Upper case letters signify differences between water-limited and control for each individual ureide and lower case letters indicate differences in the columns at $p<0.05$.

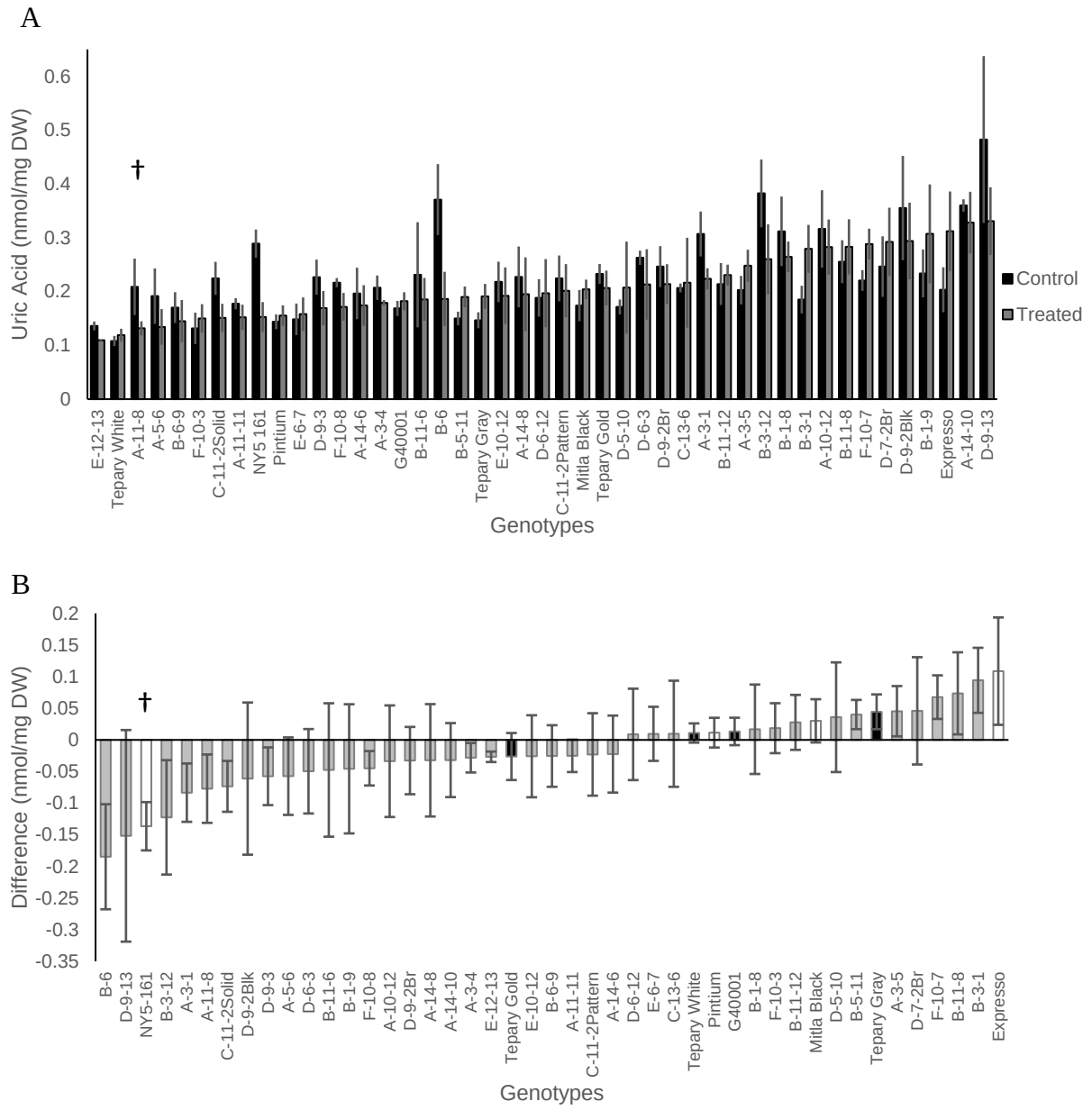


Figure 3.50: Uric Acid content in a water limitation trial in 2014. Uric acid content in leaf tissue with and without irrigation (A) and the difference (B) between the two quantities reported in (A). Uric acid was quantified in 13 tepary beans, 3 common beans, 37 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Samples reported were taken 12 days after water was discontinued. Bars in (A) show the average of 3 replicates ± standard error and bars in (B) represent the average difference between treated and control ± the propagation of standard error of the 3 treated and 3 control replicates. The tepary beans are shown in black, the common beans in white and the interpspecifics in gray. The dagger symbol (†) indicates the common bean parent, NY5-161.

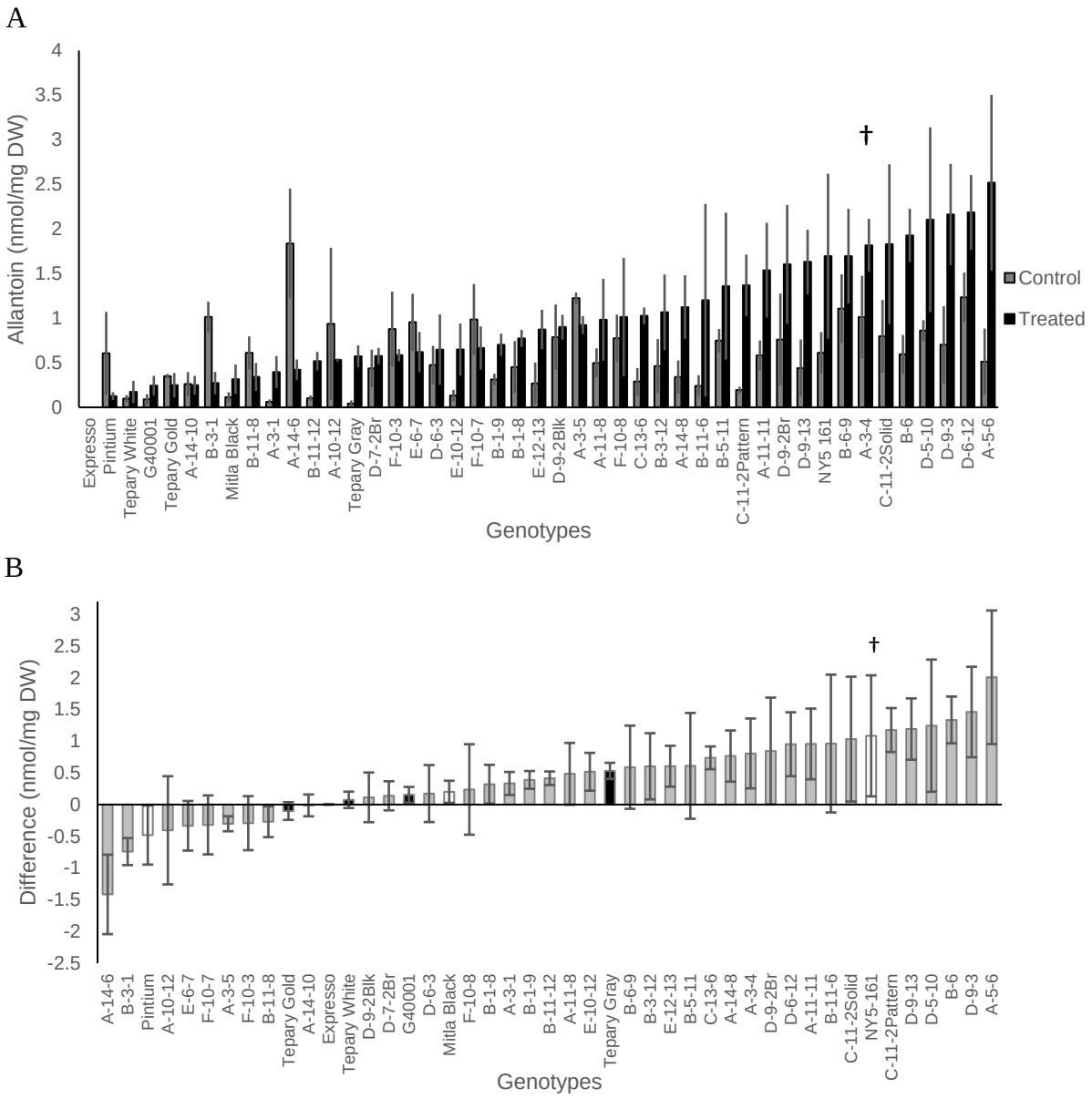


Figure 3.51: Allantoin content in a water limitation trial in 2014. Allantoin content in leaf tissue with and without irrigation (A) and the difference (B) between the two quantities reported in (A). Allantoin was quantified in 13 tepary beans, 3 common beans, 37 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Samples reported were taken 12 days after water was discontinued. Bars in (A) show the average of 3 replicates ± standard error and bars in (B) represent the average difference between treated and control ± the propagation of standard error of the 3 treated and 3 control replicates. The tepary beans are shown in black, the common beans in white and the interpspecifics in gray. The dagger symbol (†) indicates the common bean parent, NY5-161.

several genotypes had less (Figure 3.52 A). The tepary beans all accumulated allantoate, however the accumulation was lower compared to the interspecific introgression lines and common beans. The greatest difference in a tepary bean was in Tepary Gray; treated leaves contained an average of 1.40 nmol/mg DW more than control leaves. Several interspecific introgression lines and one common bean (NY5-161) accumulated much

greater levels of allantoate, with some genotypes having over 7 nmol/mg DW more allantoate in the treated leaves than the control (Figure 3.52 B). For ureide content of individual genotypes see Table D-3 and D-4.

3.10.3. Correlations between ureide quantity and agronomic traits during water limitation stress

Correlation analysis was completed on the 2013 and 2014 data to compare the ureide quantity in the leaves of each line and the yield, including both the irrigated and the stressed lines.

In 2013, correlation analysis was completed on the drought scores and ureide content was completed for the water-limited samples (Table A-31). The drought scores on day 6, day 8 and day 10 were correlated to each other (r=0.858, 0.833 and 0.792 for day 6 and 8, day 6 and 10 and day 8 and 10 respectively). However, drought scores and the ureide content were not correlated (Figure 3.53). Yield was negatively correlated to the uric acid content on day 4 (r= -0.360) (Figure 3.54). No other correlations between yield and ureide quantity were significant ($p<0.05$) even when outliers were removed. The yield difference between the stressed replicates and the non-stressed replicates was correlated with the allantoate difference between the stressed replicates and the non-stressed replicates on day 4 (r=0.530) (Figure 3.53) (for full correlations see Table A-31 and A-32).

In 2014, yield was negatively correlated with uric acid, allantoin, and allantoate (r= -0.176, -0.239, and -0.278, respectively) (Table A-36). Drought scores were positively correlated with allantoin and allantoate content (r= 0.187 and 0.236, respectively) (Figure 3.55). The difference in yield between the treated and the control plants did not correlate with the difference in ureide quantity (Table A-37).

Similar to the cold trial, correlations existed among individual ureides (for full correlation see Table A-31 and Table D-2). The amount of allantoin was highly correlated with the amount of

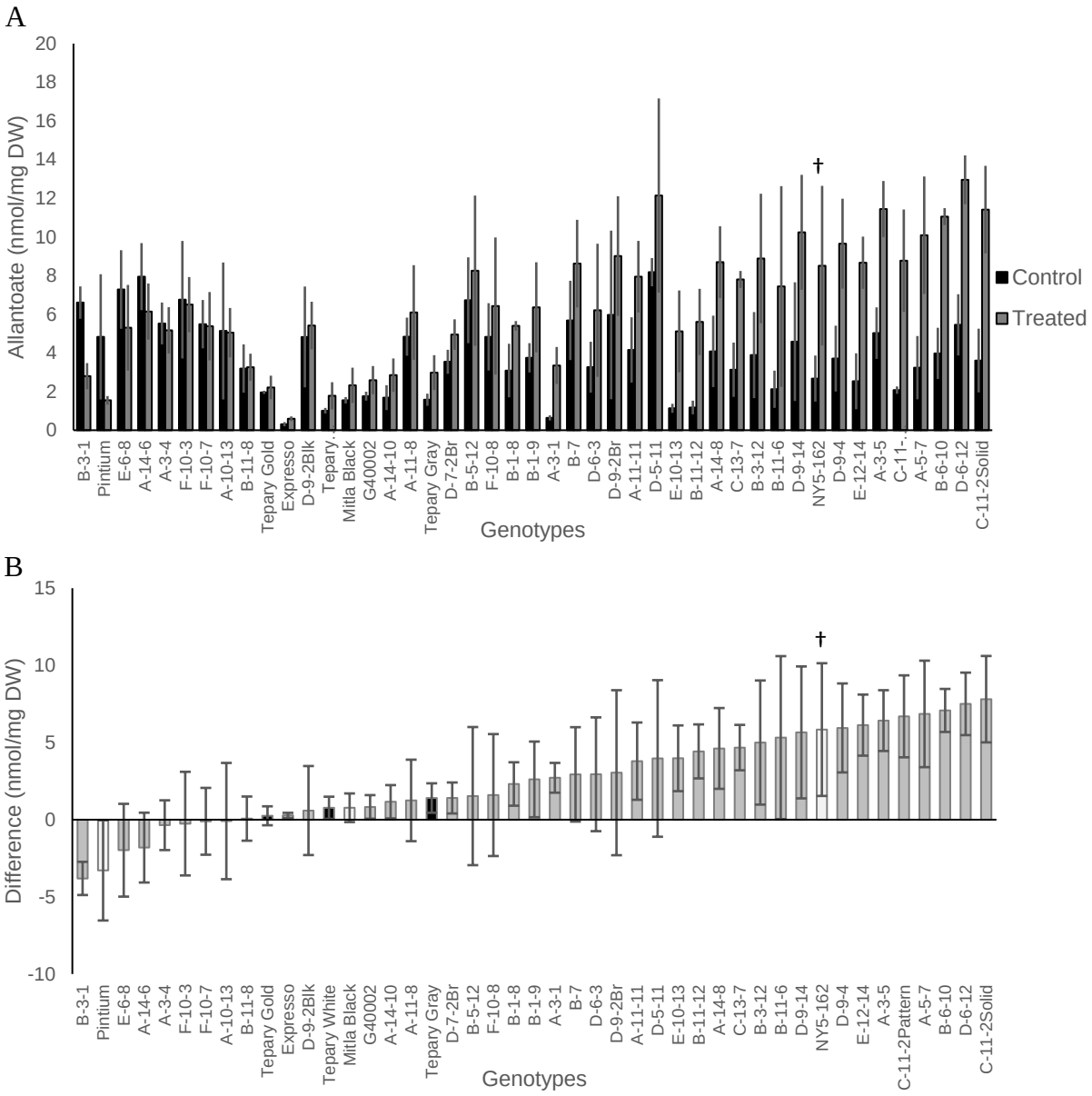


Figure 3.52: Allantoate content in a water limitation trial in 2014. Allantoate content in leaf tissue with and without irrigation (A) and the difference (B) between the two quantities reported in (A). Allantoate was quantified in 13 tepary beans, 3 common beans, 37 interspecific introgression lines and 1 accession of unknown species designation (Mitla Black). Samples reported were taken 12 days after water was discontinued. Bars in (A) show the average of 3 replicates ± standard error and bars in (B) represent the average difference between treated and control ± the propagation of standard error of the 3 treated and 3 control replicates. The tepary beans are shown in black, the common beans in white and the interpspecifics in gray. The dagger symbol (†) indicates the common bean parent, NY5-161.

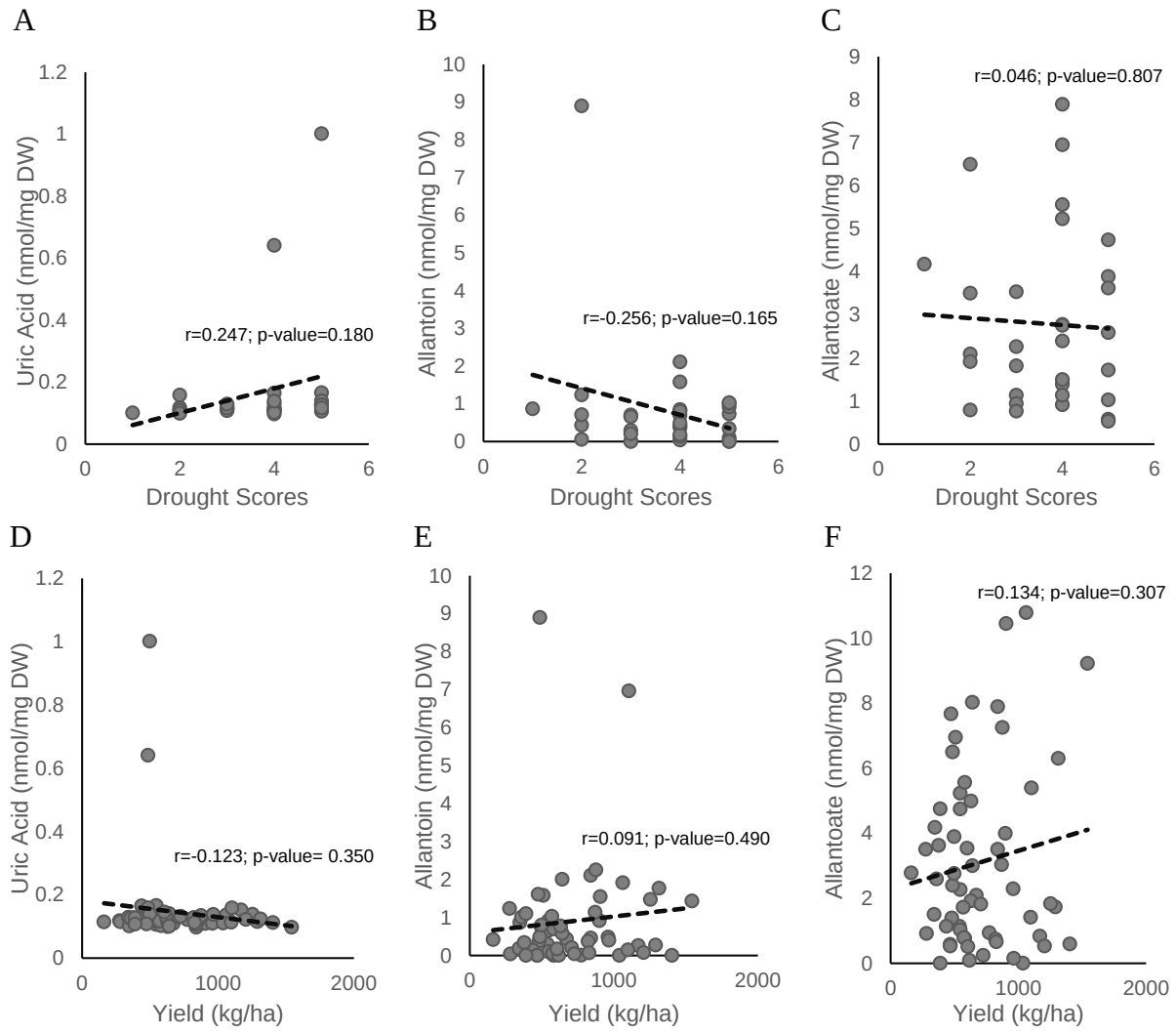


Figure 3.53: Correlations in the water limitation trial in 2013. Uric acid, allantoin and allantoate were quantified in a water limitation trial in leaves of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean. Data from 10 days after water removal is shown. Correlations depicted are between uric acid and drought scores (A), allantoin and drought scores (B), allantoate and drought scores (C), uric acid and yield (D), allantoin and yield (E), and allantoate and yield (F). No correlations in the above figures are statistically significant.

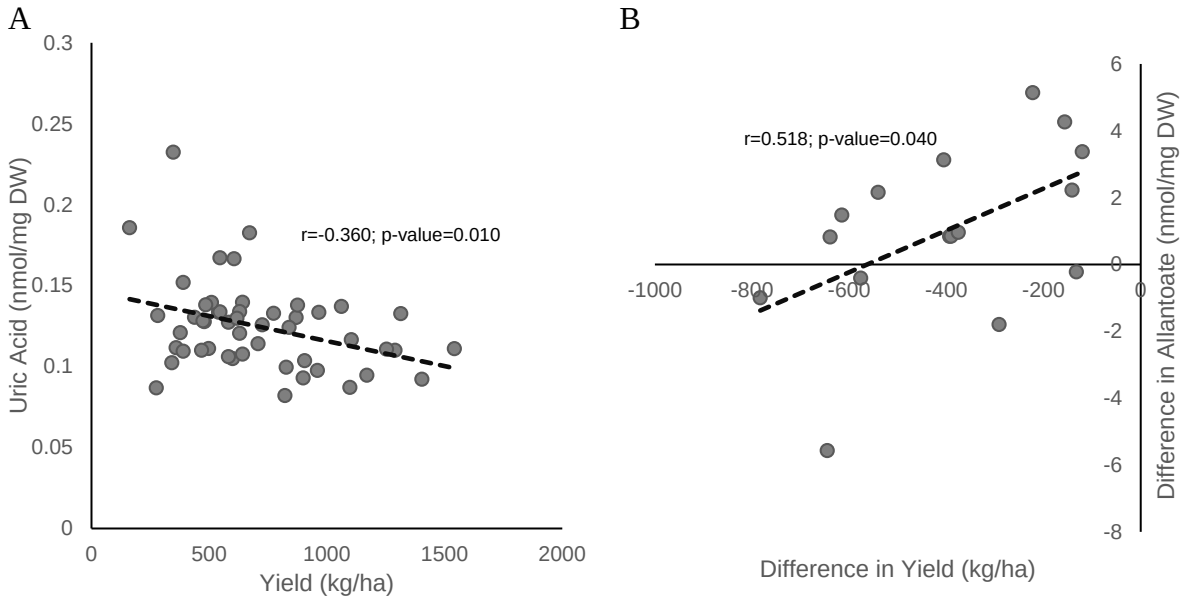


Figure 3.54: Significant correlations with yield and ureides in the water limitation trial in 2013. Uric acid and allantoate were quantified in a water limitation trial in leaves of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean. Data from 4 days after water removal is shown. The correlations depicted are between uric acid and yield (A) and the difference in allantoate content between treated and control leaves and the difference in yield between the treated and the control plants (B). These correlation are significant at $p < 0.05$.

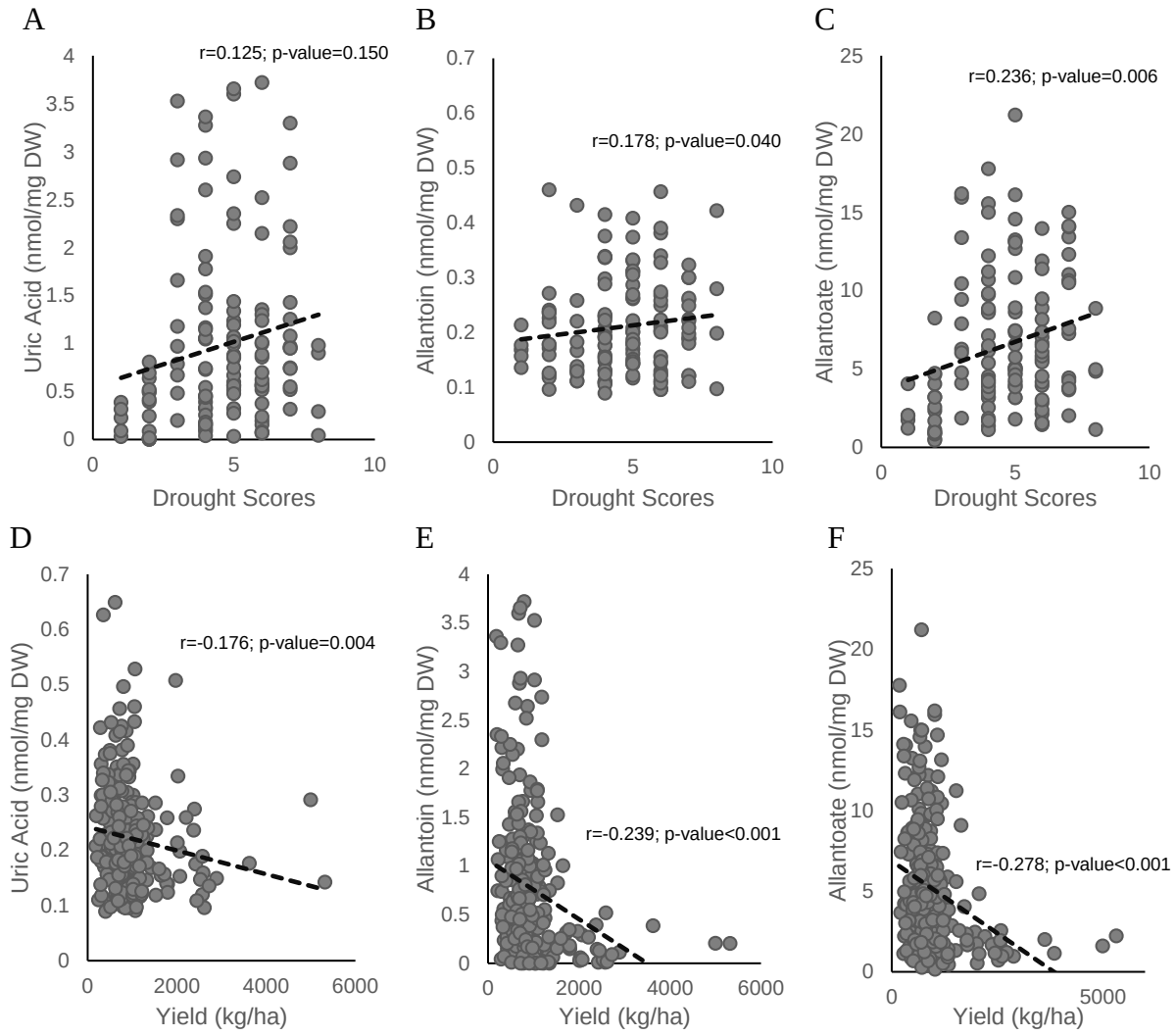


Figure 3.55: Correlations in the water limitation trial in 2014. Uric acid, allantoin and allantoate were quantified in a water limitation trial in leaves of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean 12 days after water was removed. Correlations depicted are between uric acid and drought scores (A), allantoin and drought scores (B), allantoate and drought scores (C), uric acid and yield (D), allantoin and yield (E), and allantoate and yield (F). All correlations in the above figures except for (A) are statistically significant ($p<0.05$).

allantoate in the leaf tissue, both in 2013 and 2014 (Table A-31 and A-36). No relationship between uric acid and allantoate and allantoin was significant in 2013, however, in 2014 uric acid was negatively correlated with both allantoin and allantoate (r= -0.225 and r=-0.251) (Table A-36).

3.11. Summary of field trial results

Ureides accumulated under water limitation and sub-zero temperature stress (as well as the induced oxidative stress reported in section 3.2.1) which supports the hypothesis that ureides respond to all abiotic stresses. Response in ureide content differed among genotypes, supporting the hypothesis that ureide accumulation is under genetic control. Although change in ureide content during stress had previously only been described as an increase, both increases and decreases in ureides were observed when multiple genotypes were analyzed. I hypothesized that ureide quantity during stress, or the change in the ureides content because of the stress, would correlate with the abiotic stress tolerance of a genotypes. Although some correlations were found supporting this hypothesis, the correlations were neither strong, nor universal, leading to little support based on the time-points analysed and for the method used.

Tepary beans survived the sub-zero temperature stress and had higher yields under water limitation than did common bean, supporting the hypothesis that tepary beans are more tolerant to abiotic stresses than common bean. In each trial, several interspecific introgression lines responded better than the common bean parent, indicating promise for this approach in increasing common bean stress resistance.

4. Discussion

4.1. Comparison of ureide accumulation between nitrogen-fixing plant and nitrogen-fertilized plants

4.1.1. Ureides accumulate and allantoinase activity increases in nitrogen-fixing and nitrogen-fertilized leaf tissue during water limitation

My hypothesis that ureides accumulate in nitrogen-fertilized soybean similar to nitrogen-fixing soybean during water limitation was based on the conclusion that ureide accumulation was not linked with the inhibition of nitrogen fixation (Ladrera et al., 2007) and the observation that allantoin accumulated in Arabidopsis, a non-legume, during periods of darkness and increased leaf age (Brychkova et al., 2008). I was able to show that both nitrogen-fixing and nitrogen-fertilized plants accumulate ureides over prolonged soil drying, supporting this hypothesis (Figures 3.1 and 3.2). During my thesis work, this hypothesis was also supported by data presented by Alamillo et al. (2010) in *Phaseolus vulgaris*, but in their study accumulation in non-fixing bean was only shown as total ureides at one point during drought stress.

Basal levels of allantoin and allantoate were higher in leaves of nitrogen-fixing soybean than in leaves of nitrogen-fertilized soybean (Figures 3.1 and 3.2). Nitrogen-fixing soybean plants transport nitrogen in the form of allantoin or allantoate and nitrogen-fertilized soybean plants transport nitrogen primarily as nitrate or as amino acids, the organic products of nitrate reduction (Herridge et al., 1988), therefore the difference in basal ureide content between leaves of nitrogen-fixing and nitrogen-fertilized soybean can be attributed to nitrogen fixation. The disparity in allantoin and allantoate levels between nitrogen-fixing and nitrogen-fertilized soybean was magnified during water limitation. This difference is likely due to active influx of ureides from nodules, whereas leaves of nitrogen-fertilized soybean may have a lag as the ureide catabolic pathway would not be as active. Therefore, it is likely that the sub-cellular ureide transporters (if any exist) and enzymes are limiting the amount of ureides that are accumulating. This is supported by my observation that allantoinase is more active in nitrogen-fixing tissue compared to nitrogen-

fertilized tissue (Figure 3.3), but cannot be confirmed as the activity of the other ureide enzymes and transporters is unknown.

In both control and water-limited soybean leaves, the predominant ureide in leaves of nitrogen-fertilized soybean was allantoin whereas the predominant ureide in leaves of nitrogen-fixing soybean was allantoate (Figures 3.1 and 3.2). Allantoin accumulation also became significant in nitrogen-fertilized soybean leaves earlier than allantoate accumulation, which suggests allantoin may be more important during water limitation, at least in nitrogen-fertilized soybean. In common bean, Alamillo et al. (2010) also observed more allantoate than allantoin in leaves of nitrogen-fixing plants, however the ureide content in non-fixing bean was presented as total ureides and, therefore, it cannot be determined which ureide was predominant in non-fixing bean plants following drought treatment (Alamillo et al., 2010). In research using Arabidopsis, allantoin is the predominant ureide that accumulated (Brychkova et al., 2008). Between allantoin and allantoate, allantoate may be the predominate ureide in nitrogen-fixing plants because of active ureide catabolism. The difference in the predominant ureide was coupled with leaf allantoinase being more active in nitrogen-fixing soybean compared to nitrogen-fertilized soybean and therefore converting allantoin to allantoate at a greater rate (Figure 3.3).

Alamillo et al. (2010) reported that the gene expression of allantoinase increased during stress in *P. vulgaris*, therefore allantoate accumulation was likely due to an increase in allantoate synthesis. In contrast to gene expression, allantoinase specific activity, which was only analyzed in nitrogen-fixing tissue, was not different in leaves of drought stressed plants compared to the control at the one time-point analyzed. In my work, allantoinase specific activity in leaves of nitrogen-fixing soybean was highly variable, but significantly increased both 8 and 10 days after water limitation (Figure 3.3 C,D). The increase of allantoinase activity for both nitrogen regimes was confirmed by calculating activity on an area basis (Figure 3.3 A,B). Although allantoinase activity had been previously studied in nitrogen-fertilized soybean during development (Díaz-Leal et al., 2012; Duran and Todd, 2012), research into changes in allantoinase activity in nitrogen-fertilized plants during drought has not been completed. If allantoin is the molecule acting in stress mitigation, as hypothesized (Gus'kov et al., 2004), it would be assumed that nitrogen-fertilized plants would decrease the breakdown of ureides produced by purine remobilization during stress, as there would be no need to break allantoin down to release available nitrogen. Increasing activity

of allantoinase, even in nitrogen-fertilized plants, suggests regulation of the pathway, possibly to provide another compound downstream of allantoin.

Interestingly, the change in allantoinase activity in response to drought was the same in nitrogen-fixing soybean leaf tissue as it was in nitrogen-fertilized soybean leaf tissue (Figure 3.4). This is an important observation as it suggests that the ureide stress response is a dedicated response that occurs in a similar fashion whether the plant is nitrogen-fixing or not. Irrespective of what processes are already active in the plant, the plant activated this response at the same rate, confirming a direct link between abiotic stress and ureide metabolism and accumulation. These data support previous conclusions that the ureide stress response is not dependent on nitrogen-fixation (King and Purcell, 2005; Ladrera et al., 2007) and my hypothesis that ureide accumulation during water limitation is comparable in nitrogen-fertilized soybean and nitrogen-fixing soybean. Most of the experiments reported in this thesis after the completion of this experiment used nitrogen-fertilized plants to isolate the ureide response to abiotic stress and reduce the complication of ureide metabolism from nitrogen fixation.

4.1.2. ROS induction with methyl viologen causes an accumulation of ureides but decreases allantoinase activity

Methyl viologen (MV), a redox cycling herbicide that mainly affects the chloroplast and the mitochondria, generates O_2^- which then dismutates to H_2O_2 (Vanderauwera et al., 2005). To test my hypothesis that ureide accumulation is due to an increase of ROS and that ureides scavenge the ROS produced during abiotic stress, MV was used to induce ROS production without other physiological responses that ureides may be reacting to during water limitation, such as osmotic adjustments and decreased transpiration (Mullet and Whitsitt, 1996). Allantoin and allantoate accumulated in leaves of nitrogen-fixing and nitrogen-fertilized soybean after treatment with MV, although the accumulation was variable between replicates (Figures 3.5 and 3.6). At all time-points after 12 hours, MV-treated leaves had more allantoin than the paired control, but the amounts deviated between replicates. As MV was dabbed onto leaves, coverage may not have been uniform and, therefore, levels of stress could have differed between leaves and locations on the leaf. The accumulation of allantoin observed seemed to occur earlier in leaves of nitrogen-fixing soybean than in leaves of nitrogen-fertilized soybean, which again suggests that the leaves of nitrogen-

fixing soybean have the ureide metabolism mechanisms already active and may be able to respond quicker to the change in ROS status. Alternatively, the quicker rate of accumulation in leaves of nitrogen-fixing soybean may be due to import from nitrogen fixation.

Diurnal changes may also be increasing variability in this experiment. Sampling occurred every twelve hours, with the zero time point being early after plants were exposed to light. Variation in diurnal ureide content has been previously documented in soybean petioles, with total ureide quantity doubling from early morning to late afternoon (de Silva et al., 1996; Purcell et al., 1998). Differences in individual ureide quantity were not as great in this experiment, which assayed leaf content, although variation was observed. Surprisingly, unlike Purcell et al. (1998), allantoin and allantoate in the leaves of both the nitrogen-fertilized and nitrogen-fixing soybean plants were highest at 24 and 48 hours, which would have occurred in the morning (Figure 3.5 and 3.6). Differences may be due to the different plant organs assayed (petioles versus leaf tissue).

A major difference between water limitation and induced oxidative stress was observed in allantoinase activity as MV application caused a decrease in activity instead of the increase anticipated from observations during water limitation (Figures 3.3 and 3.8). Although both MV and drought induce oxidative stress, the mechanisms they employ are different, and varying molecular responses to the stresses have been documented (Vranova et al., 2000). MV is an irreversible oxidative stress and the interaction of MV and the photosynthetic apparatus leads to extensive production of H_2O_2, whereas the stress imposed by water limitation is more localized (Taylor et al., 2005). Paraquat (active ingredient: MV) causes degradation of large numbers of enzymes (Taylor et al., 2005) and soluble protein levels decrease in isolated leaf disks after treatment with MV (Figure 3.24). Allantoinase may be an enzyme that is degraded, however, as MV generates ROS primarily in the chloroplast and allantoinase is located in the endoplasmic reticulum (Hanks et al., 1981), this is unlikely. Alternatively, allantoin may be beneficial to the plant during oxidative stress, whereas a ureide downstream of allantoin may be important to the plant during water limitation for a separate physiological reason, such as for synthesis of nitrogen-containing compatible osmolytes. Another possibility for the difference in allantoinase activity may be the differing form of cell death occurring in water limitation and induced oxidative stress. It is likely that the induced oxidative stress is leading to direct cell death (necrosis) whereas water limitation causes the plant to senesce, a process in which the main function is to recycle nutrients, including nitrogen (Wingler et al., 2005). Allantoin (in leaves from both nitrogen-fixing and

nitrogen-fertilized soybean) and allantoate (in leaves of nitrogen-fixing soybean) still accumulate after MV treatment (Figures 3.5 and 3.6), which suggests ureide accumulation is linked with response to oxidative stress.

In contrast to water limitation, where allantoinase activity increased at a similar rate (Figure 3.4), MV treatment caused a greater decrease in allantoinase activity in leaves of nitrogen-fixing soybean than in leaves of nitrogen-fertilized soybean (Figure 3.9). Methyl viologen inhibits nitrogen fixation in the nodules when roots of plants are treated (Marino et al., 2006), however as the MV was applied to the leaves, inhibition in the nodules is unlikely to be the case in this experiment. Also, as drought causes inhibition of nitrogen fixation (Weisz et al., 1985), it is likely the response to drought and MV would be the same. Therefore, as the response is opposite, differences in ureide metabolism exist between drought stress and induced oxidative stress and this affects nitrogen-fixing plants differently than nitrogen-fertilized plants. It is possible that the fertilized plants were healthier, better supplied with nitrogen and, as they had less of a carbon sink required for nitrogen fixation, they were able to utilize the carbon during the stress. Therefore nitrogen-fertilized plants could have slightly delayed the onset of the stress, however more experimentation would have to be completed to confirm this hypothesis.

The intention of this experiment was to observe the relationship between ureide accumulation and ROS production, however irreparable damage was caused to the leaf tissue and eventually caused other physiological symptoms that the ureides could potentially be responding to, such as dehydration of tissues (Figure 3.7) or damage to macromolecules. Therefore, any results observed cannot solely be attributed to ureide response to ROS.

4.2. Isolated leaf disk experiment

4.2.1. Oxidative stress decreased the ureide uptake from the solution

After a preliminary isolated leaf disk experiment demonstrated that an exogenous application of allantoin had an ameliorating effect on the damage caused by oxidative stress by MV application (Figure 3.10), a larger experiment was conducted treating leaves of nitrogen-fertilized soybean with both MV and hydrogen peroxide (H_2O_2) to act as both an exogenous and endogenous form of ROS.

After being bathed in allantoin or uric acid, the leaf disks took up the respective ureide, although it could not be confirmed which cellular compartment the ureides were in. The leaves submerged in allantoin had increased levels of allantoate, suggesting at least some of the allantoin was present in the ER, where allantoinase is located (Hanks et al., 1981). Similarly, uric acid may have been catabolized, although the increase in downstream ureides was minor. It is possible that uric acid was not translocated to the peroxisome, where it is broken down to allantoin (Hanks et al., 1981), or only a small amount entered the peroxisome. The lack of allantoate in the uric acid pre-conditioned leaves suggests that if there was an increase of allantoin it was either not completely breaking down, that allantoate was breaking down quickly and completely, or the quantity of resulting allantoate was undetectable.

As expected, the increase of ureides in the leaves was paired with a decrease of ureides in the pre-conditioning solution (Figures 3.12 and 3.20-3.32). The decrease of allantoin in its solution became significant earlier in the analysis than the decrease of uric acid. Collier and Tegeder (2012) studied an allantoin transport-deficient yeast mutant expressing the soybean genes for the ureide permease (UP), which transports ureides between cells. They reported the substrate efficiency of UP was highest for allantoin and only had marginal affinity for uric acid (Collier and Tegeder, 2012). Therefore, the disparity in uptake rate could be due to the difference in efficiency of the ureide transporters moving allantoin and uric acid in and out of the cell, however this cannot be confirmed by the data presented in this thesis.

The allantoin pre-conditioning solution that contained leaves also contained allantoate, whereas in the absence of leaves the solution contained no breakdown products (Figures 3.31 and 3.32). Therefore, either the allantoin was being metabolized in the leaf and was re-released into the solution or metabolic enzymes from the plant were released from the cell. Breakdown products of uric acid were also detected in the solution with the leaves, however the uric acid solution treated with H_2O_2 also contained allantoin in the absence of leaf tissue (Figure 3.28). Since allantoin will accumulate as a stable breakdown product of the oxidation of uric acid (Paul and Avi-Dor, 1954; Howell and Wyngaarden, 1960), at least a portion of the allantoin in the pre-conditioning solution with leaves can be accounted for by direct chemical breakdown (discussed in greater detail in section 4.2.4).

MV or H_2O_2 decreased leaf ureide levels (Figure 3.12 and 3.20). Since the breakdown of uric acid and allantoin was shown to not be a chemical reaction (with the exception of uric acid in

H_2O_2; discussed in section 4.2.4), the decrease in leaf ureide content after treatment is either due to accelerated metabolism or reduced uptake from solution. Since the treatment solution contained more ureides than the control solution, it appears that ureide uptake was reduced (Figures 3.31 and 3.32). Expression of ureide permease genes (UPs) support this prediction as the leaf tissue tended to have lower expression of UPs after MV or H_2O_2 treatment compared to the controls (Figures 3.19 and 3.27). Although ureide transporters have been characterized in Arabidopsis (Schmidt et al., 2004), soybean (Collier and Tegeder, 2012) and bean (Pelissier et al., 2004), no work has been completed to date on the changes in activity of these transporters during abiotic stress. Also, with the exception of increasing allantoin concentration amplifying the uptake of allantoin by the UPs (Schmidt et al., 2004; Collier and Tegeder, 2012), no work has been reported to date on the increasing concentration of ureides on the transcription or activity of the UPs. Allantoinase activity and expression of *ALN1* and *ALN2* decreased after treatment with MV or H_2O_2, which would be assumed to cause a buildup of allantoin if uptake or synthesis remained the same. As this was not the case, these data also support the prediction that the decreased uric acid, allantoin and allantoate in the leaf in response to MV or H_2O_2 was due to decreased uptake from the pre-conditioning solution and not an increase in metabolism in the leaf (Figures 3.15, 3.19, 3.23 and 3.27).

Since similar amounts of allantoin were present after H_2O_2 treatment or MV treatment in the allantoin pre-conditioning solution, but less allantoate was detected after treatment with H_2O_2 (Figures 3.31 and 3.32; Table B-4), H_2O_2 may have been reacting with allantoate decreasing its concentration or increasing allantoate amidohydrolase activity. To my knowledge, no reports have suggested allantoate as an antioxidant and I did not test its antioxidant properties. Exogenous allantoate, however, decreases chlorophyll degradation after treatment with H_2O_2 (Brychkova et al., 2008) therefore, study into the properties of allantoate during stress are warranted. Allantoate amidohydrolase activity decreased under drought stress in bean (Díaz-Leal et al., 2014), however as allantoinase reacted differently in my water limitation stress experiment and my induced oxidative leaf disk experiment (Figures 3.3 and 3.8), it cannot be assumed allantoate amidohydrolase activity would respond similarly between stresses. Previous work in the Todd lab has shown that allantoate amidohydrolase activity does not change during drought stress in soybean (M. Munson, personal comm.), but more experimentation would be necessary to determine the specific role of allantoate during oxidative stress.

Kaur and Halliwell (1990) observed the *in vitro* breakdown of allantoin after addition of H_2O_2 (Kaur and Halliwell, 1990). No decomposition of allantoin due to H_2O_2 occurred under the conditions used in this thesis (Figures 3.31). However, Kaur and Halliwell used a higher temperature (37°), therefore breakdown of allantoin may have been due to temperature related decomposition. Temperature did not affect the quantity of allantoin in the leaf during storage procedures (Appendix F), but may affect the chemical conversion *in vitro*.

Interestingly, the reaction to H_2O_2 differed from the MV treatment, as the amount of uric acid in the H_2O_2 treatment solution was less than the amount of uric acid in the MV treatment solution (Figures 3.31 and 3.32; Table B-4). This relationship was consistent with the results from the pre-conditioning solution that did not contain leaves (Figures 3.28), suggesting a chemical reaction occurred between uric acid and H_2O_2. It has been previously reported that uric acid is an antioxidant (Ames et al., 1981), which the data in this thesis support. It is important to note that not all studies have shown the oxidation of uric acid by H_2O_2 (Kaur and Halliwell, 1990), and specific conditions, including concentrations and time, may have to be present for the reaction to occur. As the concentration of H_2O_2 used by Kaur and Halliwell (1990) was much less than used in this thesis (1 mM of H_2O_2 compared with 40mM), the decomposition of uric acid in their study may not have been detectable. In my experiment, 40 mM H_2O_2 decreased the uric acid by approximately 10%. If the conversion was linear, 1 mM would decrease uric acid by only 0.25%, which may not have been detectible in their system.

4.2.2. Allantoin does not directly scavenge ROS and causes an accumulation of H_2O_2 in control tissue

Allantoin pre-conditioning had no effect on H_2O_2 content in the leaves, however it did decrease the H_2O_2 in the solution after both treatments (Figures 3.13, 3.21, 3.29 and 3.30). Brychkova et al. (2008) reported pre-treatment with 0.1 mM allantoin decreased the amount of O_2^- and H_2O_2 in Arabidopsis leaves after 24 hours of dark treatment (Brychkova et al., 2008). This study and my results confirm a relationship exists between allantoin and ROS when leaf tissue is present. However, these results do not show a direct antioxidant effect of allantoin, but instead show the plant responding to allantoin and decreasing ROS. In my experiments, allantoin did not decrease the amount of H_2O_2 in solution in the absence of leaf tissue (Figure 3.28), therefore the

leaf tissue was necessary to decrease the concentration of H_2O_2. My results support the conclusion presented by Wang et al. (2012) that allantoin is not a direct scavenger of ROS (Wang et al., 2012), but allantoin still has some indirect effect on the leaf that caused a decrease of H_2O_2.

Catalase activity was likely not the cause of the decreased H_2O_2 as the expression of the catalase genes did not differ between pre-conditioning solutions (Figures 3.19 and 3.27), but catalase cannot be predicted solely through analysis of transcript expression. Gus'kov et al. (2001) observed a decrease in catalase activity in mice in a hyperbaric oxygen chamber treated with an intraperitoneal injection of allantoin, as compared with the mice that were not treated with allantoin. Also, similar to the *CAT* expression data presented in this thesis, a study comparing ROS scavenging enzyme activity determined no difference in catalase activity, but greater superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity, between wild type Arabidopsis and an allantoinase (ALN) mutant Arabidopsis after treatment with cadmium (M. Nourimand, personal comm.) Likewise, allantoin stimulates SOD activity in mice after treatment in a hyperbaric oxygen chamber (Gus'kov et al., 2001). However, I saw no differences in O_2^- in response to pre-conditioning or ROS treatment, therefore no SOD activity assays were performed. Nonetheless, allantoin may be functioning in stimulating certain ROS detoxifying enzymes during abiotic stress.

Interestingly, allantoin pre-conditioning stimulated an accumulation of H_2O_2 in the control solution containing leaves (Figure 3.30). Often ROS are viewed as negative for plant performance, however at low levels H_2O_2 signals antioxidant enzymes and activates other stress response pathways and defence mechanisms (Prasad et al., 1994b; Suzuki and Mittler, 2006). Several studies have shown that the exogenous application of H_2O_2 induced abiotic stress tolerance, even though abiotic stresses themselves caused an accumulation ROS and induced oxidative stress (Ishibashi et al., 2011; Ashraf et al., 2014; Terzi et al., 2014; Hossain et al., 2015). The accumulation of H_2O_2 due to allantoin pre-conditioning may be a stress response signalled by increased allantoin content and may be inducing stress responses prior to MV or H_2O_2 addition.

4.2.3. Uric acid scavenges ROS without leaf tissue present

Uric acid has been observed to be a scavenger of ROS (Ames et al., 1981), but pre-conditioning leaves with uric acid did not affect the amount of O_2^- in the leaves after either the treatment with MV or H_2O_2 or O_2^- in the solution after MV treatment (Figures 3.13, 3.21 and 3.29).

A greater decrease in H_2O_2 may have also occurred in MV-treated leaves pre-conditioned with uric acid than those pre-conditioned with water or allantoin, but was not statistically significant (Figure 3.21). Consistent with the hypothesized role of uric acid as an antioxidant (Howell and Wyngaarden, 1960), the uric acid solution treated with H_2O_2 contained less H_2O_2 than the water solution when leaves were not present (Figure 3.28). As such, the decreases of H_2O_2 content in the leaves may have been due to uric acid scavenging that occurred in the solution thereby decreasing the stress placed on the tissue.

Since uric acid decreased in the supernatant of the uric acid pre-conditioning solution treated with H_2O_2 (Figure 3.30) and H_2O_2 decreased in the solution with uric acid when leaf tissue was not present (Figure 3.28), it is assumed that the quantity of H_2O_2 in the uric acid pre-conditioning solution containing leaves would also be lower than in the water or allantoin pre-conditioning solutions. However uric acid did not decrease the amount of H_2O_2 in the solution containing leaves. Although widely accepted as an antioxidant, under certain conditions uric acid has been shown to form free radicals and have a pro-oxidant effect (Sautin and Johnson, 2010). As these results were documented in humans, it cannot be confirmed this occurs in plants, however the possibility cannot be ruled out.

Average H_2O_2 slightly increased in the control uric acid pre-conditioning solution containing leaves (Figure 3.30), which could possibly be due to enzymatic breakdown of uric acid. Uricase, the enzyme that converts uric acid to allantoin, also yields H_2O_2 (Angermuller and Fahimi, 1986). It was expected that adding exogenous uric acid would stimulate the enzymatic breakdown of uric acid, however as leaves pre-conditioned in uric acid and water contained similar quantities of allantoin (Figure 3.12), the prediction that the increased H_2O_2 comes from amplified uricase activity is unlikely. Instead, uric acid may be stimulating the production of H_2O_2 to signal stress response, as previously discussed in section 4.2.2 with the similar response proposed with allantoin.

4.2.4. Both allantoin and uric acid decrease cell death after H_2O_2 treatment

Allantoin, although already shown not to be an antioxidant (see section 4.2.2), does have a protective role in the leaf tissue and decreases the effect of the stress on the leaf tissue, determined by the reduction of cell death after treatment with H_2O_2 and MV in allantoin pre-conditioned leaves (Figures 3.14 and 3.22). Uric acid pre-conditioning of leaf tissues did not affect cell death during the MV treatment, but did decrease cell death in the H_2O_2 treatment (Figures 3.14 and 3.22). This was the opposite relationship as what was expected, because uric acid mitigated the increase of H_2O_2 content in the MV treatment but not in the H_2O_2 treatment (Figure 3.30). However, leaves pre-conditioned in uric acid did not increase H_2O_2 levels after treatment with H_2O_2, which may indicate why less cell death occurred (Figure 3.13). However, since H_2O_2 has both harmful and helpful roles in the plant (Prasad et al., 1994b), it is possible that the H_2O_2 levels are not an appropriate indicator of cellular health.

Although data showed the decrease of cell death from pre-conditioning tissues with uric acid and allantoin during ROS treatment, it is unknown if the decrease in cell death was caused by a direct effect of these ureides on the cell membranes or an indirect effect from increasing other defence mechanisms and, in turn, decreasing the oxidative stress on the tissue. Gus'kov et al. (2001) determined that mice subjected to oxidative stress had less lipid peroxidation in erythrocytes, brain lungs, liver and kidneys if an intraperitoneal injection of allantoin was given (Gus'kov et al., 2001). Less cell death would be explained by allantoin, and potentially uric acid, decreasing lipid peroxides and, therefore, membrane damage. However in this experiment, the lipid peroxide levels did not vary between the pre-conditioning solutions, so any changes seen in electrolyte leakage were not due to differences in lipid peroxidation (Figures 3.17 and 3.25).

The total soluble protein levels decreased after treatment with endogenous and exogenous ROS (Figures 3.16 and 3.24), consistent with previous reports (Iturbe-Ormaetxe et al., 1998). No difference in this decrease was observed between the different pre-conditioning solutions after treatment with H_2O_2. However, the decrease in protein after treatment with MV may have been mitigated by pre-conditioning the tissue with allantoin (Figure 3.24).

The expression of the *Senescence Associated Receptor-like Kinase* (*SARK*) gene indicates a lower level of senescence after pre-treatment with allantoin after treatment with MV. *GmSARK* regulates leaf senescence (Li et al., 2006), therefore we would expect it to increase if the plant was undergoing senescence. Under the stress conditions presented, it was assumed that the expression

of the SARK gene would increase, however the expression decreased in almost all samples analyzed by both ROS treatments (Figures 3.19 and 3.27). Either the expression increase occurred earlier in the experiment or leaves are undergoing cell death but not senescing.

4.3. Ureide response to stress differs among bean genotypes

Since ureides were shown to participate in oxidative stress response (Figures 3.14 and 3.22; Brychkova et al., 2008; Wang et al., 2012), determining differences in the accumulation of ureides among genotypes could allow for the use of ureide content as an indicator of general abiotic stress tolerance. Previous experiments analyzed ureide accumulation in only a few genotypes of common bean (Coleto et al., 2014). One of the largest studies of ureide content analyzed 15 rice genotypes and only reported basal levels of ureides in seeds, not the change in ureides in seedlings during the stress (Wang et al., 2012). Using 108 tepary bean genotypes treated with MV I saw substantial variation among genotypes (Figure 3.33). Using a subset of these plants, ureide accumulation still varied among genotypes (Figure 3.34), however results of the two experiments were not correlated. The sub-set experiment contained both the common bean parent (NY5-161) and the tepary bean parent (W6 15578). W6 15578 had a reduced ureide content after MV treatment whereas NY5-161 accumulated ureides. From these results, I predicted that in field studies the interspecific introgression lines would accumulate ureides at a lower level than the common bean but greater than the tepary bean. Because the W6 15578 seeds did not germinate in many experiments, this hypothesis could not be confirmed. However, tepary beans usually had a lower quantity of ureides than the common bean and the interspecific introgression lines demonstrated a variation in ureide levels, including more than NY5-161 and less than some of the tepary beans (Figures 3.40 to 3.45 and 3.50 to 3.53). Tepary bean is noted to have greater tolerance to both cold stress (Balasubramanian et al., 2004a) and drought stress (Beebe et al., 2013b). The lower quantity of ureides could be due to the tepary beans experiencing less stress based on the abiotic constraints compared to the common beans.

4.4. Ureide content of field grown common bean, tepary bean, and interspecific introgression lines

4.4.1. Ureides accumulate during sub-zero temperature and water limitation stress, but not in all genotypes.

Strauss et al. (2007) quantified total ureides in leaves of two cultivars of soybean varying in chilling sensitivity and observed a decrease in leaf ureide content after chilling. These authors used nitrogen-fixing soybean plants, therefore the reduction in ureides could have been due to a decrease of ureides being transported during nitrogen fixation, which is delayed during cold temperatures (Zhang et al., 1995). In the 2013 and the 2014 trial results presented in this thesis, the three ureides measured were observed to accumulate in some genotypes after sub-zero temperature stress. However, a decrease in uric acid, allantoin, and allantoate in several genotypes was also documented in both years (Figures 3.40 to 3.45). Therefore, the decrease in total ureides shown previously could have been genotype specific. Also, as they measured total ureides and as I measured uric acid, allantoin and allantoate separately, effects of other ureides, such as ureidoglycolate, were not included in my ureide calculations and could have contributed to the decrease of total ureides reported in Strauss et al. (2007).

To date, literature from field studies focuses on the accumulation of ureides during drought stress. A similar relationship to the cold stress field study was documented in the water limitation stress field study with some genotypes, but not all, accumulating ureides (Figures 3.40-3.45 and 3.50-3.52; Table D-3). Genotypic differences in the response to exogenous ureides have been demonstrated in rice, as some genotypes responded to seed treatment with exogenous allantoin and some did not (Wang et al., 2012). As genotypic differences occur not only in the accumulation of ureides but also the response to ureides, the mechanism(s) of ureide accumulation may not be as simple as previously suggested. Therefore, previous studies that used only one or two genotypes may not be applicable as a model for every plant, even within the same species.

Basal levels of allantoin in rice grains showed a positive correlation with seedling survival of the grains (reported r^2= 0.85 in indica cultivars and 0.44 in japonica cultivars). The authors determined this relationship was due to resistance to unfavourable environmental conditions during germination (Wang et al., 2012), although additional nitrogen availability may have played a role. In my sub-zero temperature stress experiment, survival of plants after the sub-zero

temperature stress was correlated with uric acid content (Table A-26 and A-27). However, there was no significant correlation between allantoin and any measured agronomic trait. These two studies differed in two important ways. Firstly, the allantoin quantified by Wang et al. (2012) was measured in the grain before sowing whereas the ureides quantified for this thesis were measured in the leaves immediately before the stress. Second, the stress imposed in the rice study was general cold stress from being sown two weeks before normal whereas the stress imposed in this study was a harsher sub-zero temperature stress. Therefore, variation in results could be accounted for by differences in plant species, lifecycle, plant organ, severity of stress and type of stress.

In my water limitation trial, the drought stress imposed was a terminal stress and only affected the yield of the plants and not the plants' survival. Results of my water limitation trial could vary from the Wang et al. (2012) study and the sub-zero temperature study reported in this thesis. In 2013, biomass, yield and harvest index were correlated with total amount of ureides, but the data were inconsistent between days after the stress was induced (Table A-31). The 2014 data showed stronger correlations, possibly because samples were dried more promptly after removal from the plant.

Correlations, although significant, would not be strong enough to use ureides as a proxy indicator of stress tolerance in a breeding program at this stage. More research on different sampling procedures (for example, timing, plant organ, and life cycle stage) could be completed to see if modifications lead to stronger correlations.

Unsurprisingly, in all correlations completed the quantity of allantoin and allantoate were highly positively correlated, as were the changes in allantoin and allantoate during stress. However, uric acid compared to allantoin and allantoate was usually negatively correlated. In other words, if uric acid content is high, allantoin and allantoate content is low or if uric acid content is low, allantoin and allantoate content is high. Therefore, the regulation over ureide accumulation may not be controlled by or may only partially be controlled by allantoinase and allantoate amidohydolase, as has been previously suggested in common bean (Alamillo et al., 2010), but control may exist in the metabolism or movement of uric acid and other upstream intermediates. Study of the activity of uricase under abiotic stress is therefore warranted, along with the expression of the sub-cellular transporter(s), if identified.

4.5. Common bean, tepary bean and tolerance to abiotic stress

4.5.1. Change in H_2O_2 after cold bath treatment correlates to survival in the field

In other plant species, controlled environment chilling survival had been shown to be highly correlated to in field survival (Salgado and Rife, 1996; Prášil et al., 2007). In my experiment, leaves were chilled in the cold bath instead of full plant chilling, similar to methods used by Martinez Rojo (2010) who compared leaf disk electrolyte leakage in bean to whole plant freezing and concluded leaf disk electrolyte leakage can be an effective initial method to evaluate the levels of tolerance to low temperature exposure stress (Martinez Rojo, 2010). Electrolyte leakage, change in O_2^- and change in H_2O_2 varied among genotypes tested for this thesis (Figures 3.35 and 3.36). A positive correlation between change in O_2^- and electrolyte leakage indicates, as expected, the greater the O_2^- evolution, the greater the difference of cell death in response to cold. Interestingly, the opposite relationship existed with change in H_2O_2, which was negatively correlated with cell death. Similar to the observations and discussion in the induced ROS experiment (Figure 3.30) an increase in H_2O_2 may not have a detrimental effect but act as a signal for stress mitigating pathways (Hossain et al., 2015).

The electrolyte leakage measurements from the cold bath experiments did not statistically correlate with the survival in the field, however changes in H_2O_2 in the cold bath did correlate with the survival of plants in both of the 2014 field frost events. Although correlations were strong ($r=0.67$ and $r=0.59$, for the first and second frost event, respectively), these data only included 15 genotypes and a larger experiment should be completed to determine if this relationship is significant in a larger population. Common bean has not demonstrated the ability to acclimate to the cold (Allen and Ort, 2001) and increasing H_2O_2 is one of the cellular responses during acclimation (Prasad et al., 1994b). Although other acclimation responses are not occurring before the stress in common bean, it is possible that H_2O_2 is important in stress response signaling pathways. O_2^- and H_2O_2 were negatively correlated in this experiment, which may suggest the genotypes with greater increase in H_2O_2 had a better system for detoxifying O_2^- and therefore less cellular stress was imposed from the cold event.

4.5.2. Tepary is superior to common bean during sub-zero temperature stress

Much of the research in cold tolerance in legumes has been in examining cold stress during germination (Hucl, 1993; Zaiter et al., 1994), or at flowering, early pod formation and seed filling stages, which are the most susceptible stages of chilling in legumes (Maqbool et al., 2009), or the overwintering of forage legumes (Badaruddin and Meyer, 2001). However, establishment failure due to early seedling freezing temperatures is hazardous to legume production (Badaruddin and Meyer, 2001), especially in bean as the growing point is above the ground and the plant will not regrow after damage. After the sub-zero temperature events, less than 30% of the common bean checks in my population survived the stress (Figures 3.38 and 3.39), therefore any increase in common bean tolerance to sub-zero temperatures at the seedling stage would be beneficial.

Balasubramanian et al. (2004a) studied wild relatives of common bean and survival to sub-zero temperature stress. Although the one tepary bean accession studied did not exhibit survival at the lowest temperatures withstood by the other wild relatives of common bean (including *Phaseolus filiformis*, *Phaseolus angustissimus*, and *Phaseolus ritensis*), it still survived temperatures lower than that of the three common beans studied (Balasubramanian et al., 2004a). The three common beans used included two accessions originally collected from high altitudes (G11031 and G23457) and CDC Blackhawk, which was previously shown to have tolerance to suboptimal temperatures during emergence (Balasubramanian et al., 2004b). Later, two other tepary accessions were documented to have greater tolerance than common beans studied (Martinez-Rojo et al., 2007). I determined tepary beans have a higher tolerance to sub-zero temperature stress under field conditions (Figures 3.38 and 3.39). The response of the genotypes studied varied, but in three frost events the survival of tepary beans as a group had a higher mean percentage survival than the common beans (for example, 35% of tepary beans survived seven days after the 2015 stress whereas only 21% of the common beans survived). The temperatures documented by Balasubramanian et al. (2004a) were lower than the temperatures the seedlings were subjected to in this study. However, the greater tolerance they observed is not surprising because other environmental factors play a role in the severity of a stress in the field, including wind and light. They also may have observed superior performance as they were only looking at leaf effects whereas survival in this study was dependent on all plant organs. As well, the leaflets

used were from 30 day old plants, whereas the seedlings analyzed in my study were at the first trifoliate stage. The unifoliate leaf and the fist trifoliate leaves were found to be the most sensitive to sub-zero temperature stress in common bean (Meyers and Badaruddin, 2001), therefore leaf age may have also contributed to differences in survival temperatures.

The population survival varied between the four trials analyzed (Figures 3.38 and 3.39; Table 3.1), which was not surprising as it has been documented that tolerance measurements vary due to the intensity and duration of the stress, the rate of stress development and the phenological timing of the stress (Srinivasan et al., 1998). The life-stage was similar between the experiment years, however the intensity, duration and rate of the stress varied between years (Table 3.1).

The importance of a long observation period during sub-zero temperature experiments was shown in this analysis. It has been noted that in plants with little to no freezing tolerance, the morning after the frost the injured plant looks flaccid and water soaked as cell membranes have lost their semipermeability and the intracellular compartmentalization is destroyed (Burke et al., 1976); the plants are not dead, but will eventually die due to the symptoms. Regrowth after sub-zero stress events differs between temperatures and ages of bean (Meyers and Badaruddin, 2001). When determining sub-zero temperature stress tolerance, completing the lifecycle is the goal. In my trials, seedlings were often still green one day after stress but had succumbed to their injuries seven days after the stress (Figures 3.38 and 3.39). This was also reflected in percent survival as average survival of the population was lower seven days after stress than immediately after stress. This occurrence has been previously noted, especially with tepary beans as some hypothesize that tepary beans rely on stress postponement rather than tolerance (Rao, 2001). This occurrence was during drought stress, but could be playing a factor in sub-zero temperature stress as well.

4.5.3. Tepary is superior to common bean during water-limited conditions

At least two tepary bean accessions with superior tolerance to drought than the common beans studied were identified, in an experiment that compared yield under irrigation and yield under rain-fed (water-limited) conditions (Rao et al., 2013). In the data presented in this thesis, tepary beans were shown to exhibit greater yield than common bean after terminal water limitation stress. In 2013, common bean had greater irrigated yield, however in 2014 and 2015, tepary beans were superior under both conditions (Figures 3.47 A, 3.48 A and 3.49 A). Tepary beans often did

better under the mild to moderate stress conditions than the irrigated conditions, suggesting either excessive irrigation hampers tepary bean performance or stress signals for the control of indeterminacy of tepary bean to increase reproduction. To the best of my knowledge, no data have been published analyzing tepary bean's response to water logging, although high water availability could cause excessive vegetative growth in indeterminate plants (De Costa et al., 1997), a growth habit that the tepary beans in this trial represented. Also, it has been noted that indeterminacy enhanced adaptation under drought conditions. In one study, indeterminate bean cultivars had greater seed yield under drought than the determinate cultivars (Rosales-Serna et al., 2003). Indeterminate bean cultivars demonstrated greater yield stability than determinate bean cultivars in an experiment of forty-two trials, differing in climate and soil conditions, over a five year period in Michigan (Kelly et al., 1987). Plants with indeterminate growth habits have been noted to have an inherent flexibility in quantity of flowers formed (Nleya et al., 1999) which allows the plant to take advantage of opportune times for growth and reproduction while delaying these processes in inopportune times. Similar terminal drought stress observations have been documented in other plants, like faba bean, as it has been determined that the greater flexibility in lifecycle can exploit mid-season water inputs (De Costa et al., 1997).

4.5.4. Traits of both common bean and tepary bean are present in the interspecific introgression population, as well as transgressive segregation

The hypothesis that tepary bean can be used to transfer genes encoding abiotic stress tolerance traits into common bean was supported by several interspecific introgression lines having superior tolerance than the common bean parent to sub-zero temperature stress (Figures 3.36, 3.38 and 3.39) and water limitation stress (Figures 3.47-3.49). However, the agronomic traits and stress response of the interspecific introgression lines varied greatly among the hybrids. Some interspecific introgression lines performed well, but there was a lot of transgressive segregation observed in this population. This was consistent with hybrid populations where extreme traits have been observed to be the norm, with 91% of studies reporting at least one transgressive trait (Rieseberg et al., 1999). Unfortunately, in this population the transgressive segregation observed was mainly in the form of undesirable traits, for example, less cold and drought tolerance than either parent (Figures 3.38, 3.39 and 3.47-3.49).

The interspecific introgression lines used in these experiments were backcrossed to the common bean parent twice. Therefore, if genetic donation was equal between parents, only 12.5% of the interspecific introgression line genome would be of tepary origin and 87.5% would be of common bean origin. The tepary-specific genes would be even less due to tepary bean and common bean having a common ancestor and significant blocks of shared loci. Therefore, it was not surprising that a large portion of the interspecific introgression population was phenotypically very similar to the common bean parent.

The tepary parent of the interspecific introgression lines (W6 15578) was a wild accession and likely contributed many traits problematic to domestic production. For example, W6 15578 did not germinate unless scarified. Potentially, this may have led to germination difficulties in offspring. As W6 15578 did not germinate in many of these experiments, determining if unsuitable traits were from the tepary parent was not possible.

Interspecific introgression lines between common bean and its wild relatives, including tepary bean, are a promising method of increasing stress tolerance in common bean (Porch et al., 2013). Although the top twenty interspecific introgression genotypes in this study were not consistent between the stresses and the years (Table E-1), several genotypes were notable and will be used in future crosses. For example, B-7-7Br and D-13-4Blk were documented to have high levels of drought tolerance in all years; D-7-2Br, E-6-3, A-11-7, and B-5-11 show high yield stability (geometric mean) over all years; and A-3-5 had superior survival after sub-zero temperature stress in all years. D-13-4Br, E-6-2, B-1-4 and D-6-13 also show promise as tolerant to sub-zero temperature stress with high rankings in at least two of the three years.

With phenotypic information gained during this study on the available cultivated tepary bean population, such as yield potential and abiotic stress tolerance, future interspecific introgression populations could be developed with a more suitable tepary bean parent and, therefore, have a greater chance of success. Of specific interest are the genotypes Tepary Gray and Tepary Gold that were higher yielding in both control and water-limited conditions and had superior survival after sub-zero temperature stress than the common bean checks, the interspecific introgression lines and most of the other tepary beans studied.

4.6. Predicted mechanism of ureides mitigating abiotic stress damage

The research presented in my thesis, along with other studies, show that allantoin does not directly scavenge ROS, but mitigates the oxidative damage indirectly. Allantoin decreases chlorophyll breakdown, H_2O_2 levels, and O_2^- levels in leaf disks in response to dark treatment (Brychkova et al., 2008). Allantoin also causes increases in proline content in rice seedlings from seeds treated with allantoin (Wang et al., 2012). The report that exogenous allantoin decreased chromosomal aberrations caused by H_2O_2 in onion seedlings (Gus'kov et al., 2002) and the data presented in my thesis documented a decrease in cell death after pre-conditioning with allantoin. Although all of these observations could be accounted for by a reduction in ROS, none of these observations prove allantoin directly reduces ROS. In Arabidopsis, allantoin accumulation, caused by either an *aln* mutant or exogenously added allantoin, activates abscisic acid (ABA) production (Watanabe et al., 2013). Increased ABA leads to activation of stress moderating pathways in the plant, therefore allantoin may indirectly decrease ROS levels through activation of other stress mitigating reactions, such as antioxidant response (Figure 4.1).

In contrast, uric acid does chemically react with H_2O_2, leading to a reduction in cell death. Uric acid is a scavenger of singlet oxygen and is oxidized by hydrogen peroxide in the presence of hematin or methemoglin (Ames et al., 1981). In this study, uric acid decreased the change in H_2O_2 after MV treatment and directly scavenged the H_2O_2 when leaves were not present (Figure 4.1). A major source of ROS is the metabolism that occurs in the peroxisome (Tripathy and Oelmüller, 2012). Since uric acid is synthesized in the cytosol and moves to the peroxisome (Hanks et al., 1981), it is likely that uric acid has the potential to act as an antioxidant in the plant.

During treatment of both isolated leaf disks and attached plant leaves, a decrease in allantoinase activity and allantoinase gene expression was observed after treatment with ROS, which may have indicated the leaf tissue is programmed to increase allantoin availability during stress. Allantoinase activity also decreases after treatment with cadmium and expression decreases in high light in Arabidopsis (M. Nourimand and S. Irani, personal comm.) This relationship was opposite in water limitation, as allantoinase activity increases. It is possible that the down-stream intermediates (allantoate, ureidoglycolate, ureidoglycine and glyoxylate) are more beneficial to the plant during water limitation. Also, it is possible that under water limitation allantoinase is not responding to ROS but for the general remobilization of nitrogen.

At the beginning of my study, I hypothesized allantoin had the role of a ROS scavenger during abiotic stress (Figure 4.2 A). The results of my thesis have demonstrated this is not the case. However, allantoin does have a positive effect on leaf tissue during stress. Therefore, one of three alternative possibilities exist on the role of allantoin during stress. First, allantoin may decrease the severity of the stress on the plant (Figure 4.2 B). There are several possibilities of how this occurs, but the most likely would be allantoin acting as a compatible osmolyte similar to several other nitrogen rich compounds (Yancey, 2005). Alternatively, allantoin may function in decreasing the ROS produced by the stress (Figure 4.2 C). The production of other metabolites, such as glycerol, requires reduction and therefore contributes to a cellular redox balance (Yancey, 2005), however this hypothesis is unlikely as NADH is created during allantoin synthesis, not used (Werner and Witte, 2011). Finally, allantoin may stimulate ROS scavenging after the ROS is produced (Figure 4.2 D), likely through the activation of SOD or APX, which has been documented in Arabidopsis during cadmium stress (M. Nourimand, personal comm.).

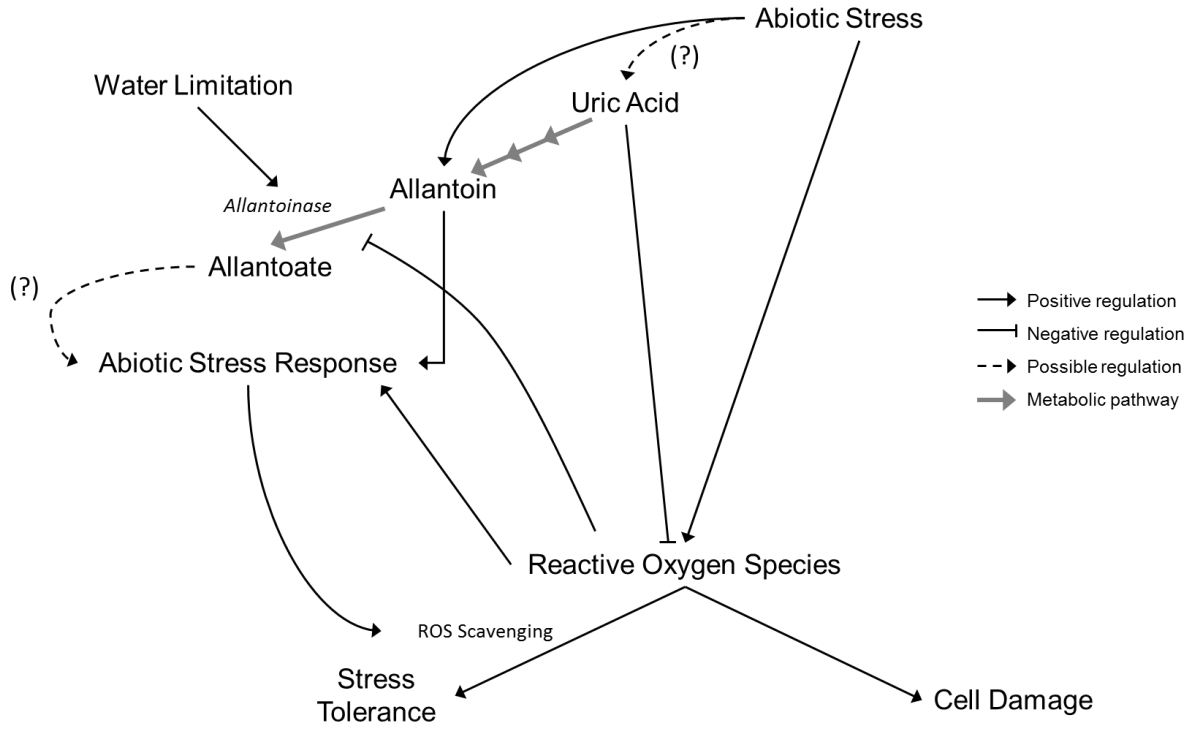


Figure 4.1: Mechanism for abiotic stress mitigation by ureide accumulation. Possible model of the effects of ureide accumulation on abiotic stress tolerance.

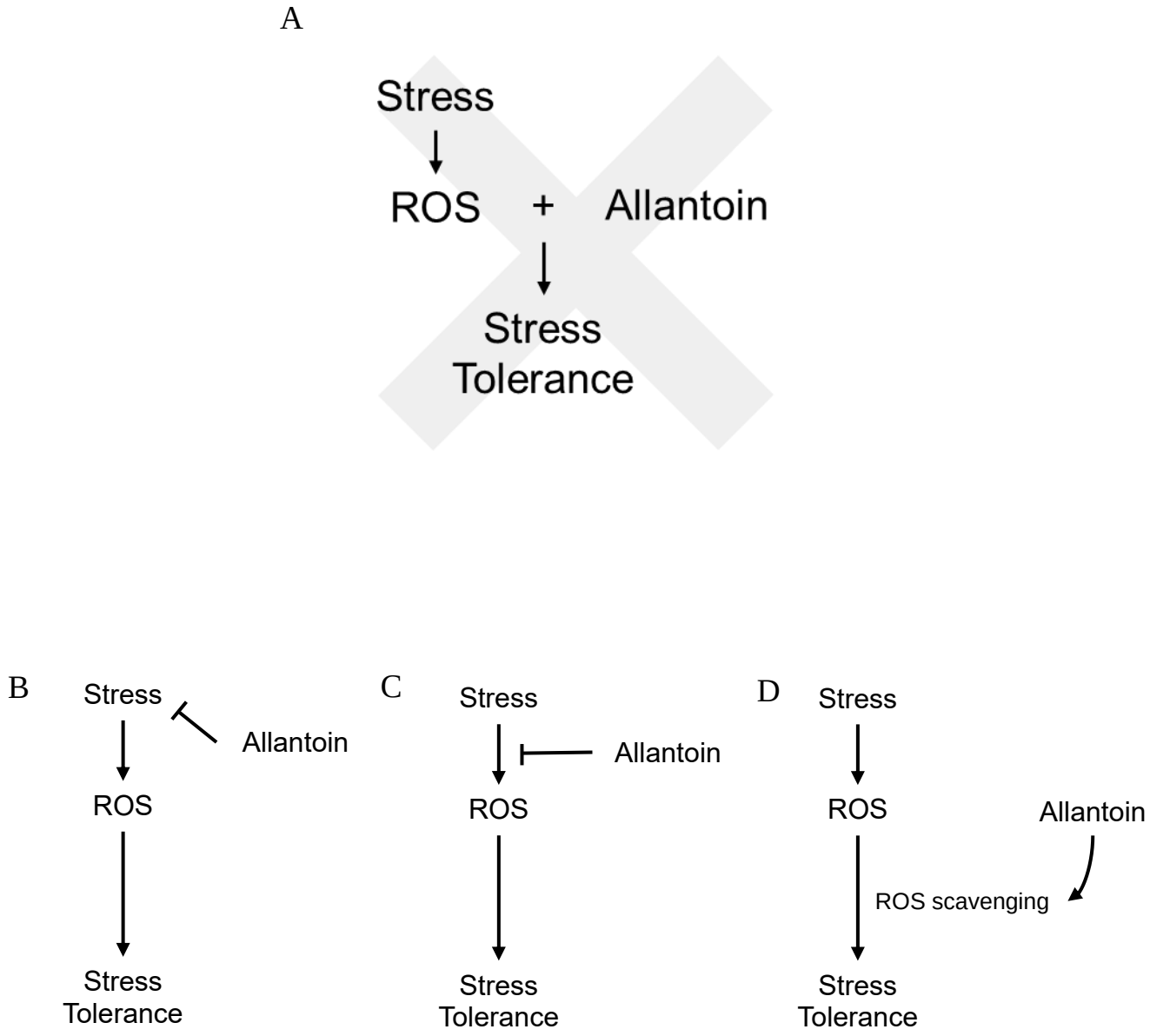


Figure 4.2: Possible functions of allantoin during abiotic stress. (A) The previous hypothesis of allantoin acting as ROS scavenger to detoxify ROS and impart stress tolerance on the plant. (B,C,D) Alternative hypothesis of the role of allantoin during abiotic stress, including (B) allantoin decreasing the stress, (C) allantoin decreasing the ROS produced after the stress, or (D) allantoin stimulating ROS scavenging after the ROS is produced by the stress.

5. Conclusions and Future Directions

Ureides, specifically allantoin and allantoate, accumulated in leaves of both nitrogen-fixing and nitrogen-fertilized soybean. The accumulation was coupled with an increase in allantoinase activity and the change in allantoinase activity was the same between nitrogen-fixing and nitrogen-fertilized soybean plants. The similarities between nitrogen-fixing and nitrogen-fertilized plants confirms a common mechanism for ureide accumulation that was not linked to nitrogen fixation. Ureides also accumulate during induced oxidative stress, suggesting a relationship between ureides and ROS. These results support my hypotheses that ureides accumulate during water limitation in nitrogen-fertilized soybean in a manner similar to nitrogen-fixing soybean and that ureide accumulation occurs in response to an increase of ROS. As this thesis shows differences in responses and functions of individual ureides during abiotic stress, determining the exact role, if a role exists, of each specific ureide in response to abiotic stress would be beneficial for more targeted research and allow for more specific analysis of what is occurring in the plant with regards to ureide accumulation and abiotic stress. Also, this thesis provides evidence that control of ureide accumulation in legumes may, at least in part, be determined by enzymes upstream of allantoinase and the transport of ureides around the cell and between the cells. Other transporters need to be discovered and characterized, if they exist, and activity of these transporters during abiotic stress needs to be researched.

Allantoin and uric acid mitigated abiotic stress damage in soybean leaves. Uric acid decreased H_2O_2 in solution and may act as an antioxidant in plants. Allantoin, however, is not an antioxidant and its association with decreases of ROS during abiotic stress occurs due to some other indirect relationship, such increasing activity of ROS degrading enzymes or signalling stress mitigation pathways. My hypothesis that ureides scavenge the excess ROS produced during abiotic stress is supported by the uric acid results, but not the allantoin results. The addition of allantoin and uric acid provided stress mitigating properties, which was evident by a reduction in cell death after exogenous and endogenous oxidative stress. These data support my hypothesis that increased ureide content has a beneficial effect on cellular integrity and will mitigate damage in response to ROS. New research is examining whether spraying solutions onto field crops (such as pyrabactin, which mimics ABA, or H_2O_2 to stimulate the stress response) is a viable option for reducing effects of abiotic stress (Park et al., 2015; Ishibashi et al., 2011). Allantoin may be a candidate for such

application or as a seed treatment as both methods of exogenous application have shown to be promising (Wang et al., 2012) and should be examined to a greater extent in the future.

I also hypothesized that ureide accumulation under sub-zero temperature stress and water limitation differs among genotypes in a population and the variation correlates with the differences in abiotic stress tolerance among the genotypes, and therefore ureide content could be used as a proxy indicator for abiotic stress tolerance. Ureide content varied among individuals within a population of tepary beans, common beans and interspecific introgression lines of tepary and common bean. However, the hypothesis that ureides could be used as a proxy indicator for abiotic stress tolerance was not supported because although correlations exist between ureides and abiotic stress tolerance, the correlations were not strong to support use in breeding programs. Different timing, plant parts, populations and extraction methods could be examined.

Data collected during the water limitation trial adds to the increasing knowledge base of drought tolerance in bean, specifically by supporting arguments that indeterminate accessions may be advantageous in areas with chronic water limitation issues. Although drought tolerance is widely studied in common bean, breeding for and knowledge of sub-zero temperature stress in common bean is limited. Planting genotypes later in the year and guaranteeing a frost event will occur at a seedling stage proved to be a valuable way to determine seedling frost tolerance. Using electrolyte leakage to determine frost stress tolerance in the field was not as successful in these experiments as in the literature. The possibility of using change in H_2O_2 content as an indicator was proposed, but needs to be verified in a larger trial.

Using tepary beans as donors of genes encoding abiotic stress tolerance in common bean is promising. The tepary beans studied demonstrated greater yield under conditions of water limitation and greater survival after a sub-zero temperature event than common beans. Some interspecific introgression lines showed promise which supports my hypothesis that tolerance genes can be introgressed from tepary bean into common bean, but many of the hybrids lacked the necessary agronomic traits for production. The population examined in this thesis will be useful in the breeding program, but with the knowledge gained through this thesis, new crosses with more appropriate tepary parents can be completed. Using these new parents in the crosses will maximize the likelihood of success in the resulting population and less work would have to be done to breed in necessary agronomic traits for production.

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7. Appendices

APPENDIX A- Statistical analysis results

Included in this appendix are supplementary statistical data for the results presented in Section 3. Tables include the F values from the one- and two-way ANOVAs, along with an indication of the significance. Correlation tables include the r values and the p values of the results of the Pearson correlational analyses. Any exceptions are indicated in the footnote of the tables, along with the degrees of freedom in the experiment and the transformation used on data that were not normally distributed.

Table A-1: Accumulation of allantoin in soybean leaves after water limitation treatment.

Source of Variation	Degrees of Freedom	Nitrogen-Fertilized		Fixing	
		Allantoin		Allantoin	
		LD	FW	LD	FW
Day	6	1.88	3.28**	10.39***	8.33***
Treatment	1	35.13***	31.77***	81.14***	63.95***
Day*Treatment	6	1.69	3.23**	7.64***	6.61***

Asterisks indicate p-value: <0.05*, <0.01**, <0.001***.

Table A-2: Accumulation of allantoate in soybean leaves after water limitation treatment.

Source of Variation	Degrees of Freedom	Nitrogen-Fertilized		Fixing	
		Allantoate		Allantoate	
		LD	FW	LD	FW
Day	6	1.70	1.80	7.72***	7.58***
Treatment	1	18.37***	11.98***	59.19***	51.56***
Day*Treatment	6	1.50	1.76	5.69***	5.92***

Asterisks indicate p-value: <0.05*, <0.01**, <0.001***.

Table A-3: Allantoinase activity in soybean leaves after water limitation treatment.

Source of Variation	DF	Nitrogen-Fertilized			Fixing		
		LD	FW	Protein	LD	FW	Protein
Day	6	1.89	1.63	0.56	3.85^{**}	5.62^{***}	2.89^{*}
Treatment	1	24.93^{***}	29.22^{***}	15.57^{***}	19.44^{***}	24.82^{***}	8.27^{**}
Day*Treatment	6	2.7^{*}	3.6^{**}	1.39	1.03	2.73^{*}	0.42

Results for two-way ANOVAs of the log of the dependent variable values. Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-4: Comparison of allantoinase activity in soybean leaves after after a water limitation treatment.

Source of Variation	Degrees of Freedom	Comparison between Fixing and Nitrogen-Fertilized		
		LD	FW	Protein
Time	6	5.35^{***}	8.31^{***}	3.66^{**}
Treatment	1	0.88	0.57	0.03
Time*Treatment	6	0.84	1.34	0.75

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-5: Accumulation of allantoin in soybean leaves after treatment with methyl viologen.

Source of Variation	Degrees of Freedom	Nitrogen-Fertilized		Fixing	
		Allantoin		Allantoin	
		LD	FW	LD	FW
Time	6	2.27	3.18^{*}	1.37	1.9
Treatment	1	11.05^{**}	15.77^{***}	10.14^{**}	13.52^{**}
Time*Treatment	6	1.95	2.81^{*}	1.51	1.81

Asterisks indicate p-value: <0.05*, <0.01**, <0.001***.

Table A-6: Accumulation of allantoate in soybean leaves after treatment with methyl viologen.

Source of Variation	Degrees of Freedom	Nitrogen-Fertilized		Fixing	
		Allantoate		Allantoate	
		LD	FW	LD	FW
Time	4	1.53	1.6	0.57	1.03
Treatment	1	6.09*	8.45**	2.48	7.29*
Time*Treatment	4	1.08	1.12	.73	1.29

Asterisks indicate p-value: <0.05*, <0.01**, <0.001***.

Table A-7: Allantoinase activity in soybean leaves after treatment with methyl viologen.

Source of Variation	DF	Nitrogen-Fertilized			Fixing		
		LD	FW	Protein	LD	FW	Protein
Time	4	5.21**	2.51	1.08	0.99	1.73	0.92
Treatment	1	18.75***	4.21*	6.81*	20.51***	18.09***	10.71**
Time*Treatment	4	1.09	0.28	0.28	1.47	1.91	0.76

Results for two-way ANOVAs of the log of the dependent variable values. Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-8: Comparison of allantoinase activity in soybean leaves after treatment with methyl viologen.

Source of Variation	DF	Comparison between Fixing and Nitrogen-Fertilized		
		LD	FW	Protein
Time	4	9.85***	5.19**	4.00**
Treatment	1	1.90	6.20*	8.2**
Time*Treatment	4	0.36	1.04	1.35

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-9: Fresh weight of leaf disks analyzed after treatment with methyl viologen.

Source of Variation	Degrees of Freedom	Nitrogen-Fertilized Tissue Weight	Fixing Tissue Weight
Time	4	4.76**	3.31*
Treatment	1	85.68***	38.86***
Time*Treatment	4	5.16**	2.99*

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-10: Superoxide accumulation in leaf tissue of isolated leaf disks during induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Superoxide Content	
		MV	**H_2O_2**
Pre-Conditioning	2	0.45	0.06
Treatment	1	1.50	1.13
Pre-Conditioning*Treatment	2	0.03	0.37

Source of Variation	Degrees of Freedom	Percent of Control		Difference from Control	
		MV	**H_2O_2**	**MV**	**H_2O_2**
Pre-Conditioning	2	0.09	0.05	0.06	0.05

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-11: Superoxide accumulation in supernatant of isolated leaf disks during induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Superoxide Content	
		MV	**H_2O_2**
Pre-Conditioning	2	0.74	1.09
Treatment	1	116.32***	57.01***
Pre-Conditioning*Treatment	2	0.98	0.29

Source of Variation	Degrees of Freedom	Percent of Control		Difference from Control	
		MV	**H_2O_2**	**MV**	**H_2O_2**
Pre-Conditioning	2	0.12	2.26	0.04	0.27

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-12: Hydrogen peroxide accumulation in leaf tissue of isolated leaf disks during induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Hydrogen Peroxide Content			
		MV		H_2O_2	
Pre-Conditioning	2	0.04		0.04	
Treatment	1	0.95		16.98^{***}	
Pre-Conditioning*Treatment	2	0.20		0.08	
Source of Variation	**Degrees of Freedom**	**Percent of Control**		**Difference from Control**	
		MV	**H_2O_2**	**MV**	**H_2O_2**
Pre-Conditioning	2	4.32^{*}	1.23	3.33	0.84

Distribution was assessed and data for both two-way ANOVAs was log transformed, as well as H_2O_2 treated leaf tissue analyzed as a percentage of its control before a one-way ANOVA. Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-13: Hydrogen peroxide accumulation in supernatant of isolated leaf disks during induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Hydrogen Peroxide Content			
		MV		H_2O_2	
Pre-Conditioning	2	1.43		0.69	
Treatment	1	31.37^{***}		26.43^{***}	
Pre-Conditioning*Treatment	2	4.63^{**}		4.71^{**}	
Source of Variation	**Degrees of Freedom**	**Percent of Control**		**Difference from Control**	
		MV	**H_2O_2**	**MV**	**H_2O_2**
Pre-Conditioning	2	4.64^{*}	4.35^{*}	3.48^{*}	4.16^{*}

The hydrogen peroxide levels in the MV treatment were transformed by taking a square root before analysis by two-way ANOVA. The percent of control of the MV and HP treatments were log transformed prior to analysis. Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-14: Electrolyte leakage of isolated leaf disks during an induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Electrolyte Leakage	
		MV	H_2O_2
Pre-Conditioning	2	3.77*	1.4
Treatment	1	131.71***	98.86***
Pre-Conditioning*Treatment	2	6.33**	5.48**

Source of Variation	Degrees of Freedom	Percent of Control		Difference from Control	
		MV	H_2O_2	MV	H_2O_2
Pre-Conditioning	2	4.12*	3.61*	4.76*	5.22**

The methyl viologen and hydrogen peroxide treatment shown as a percent of control was log transformed before analysis by one-way ANOVA. Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-15: Allantoinase activity per leaf disk in isolated leaf disks during an induced oxidative stress treatment.

Source of Variation	Degrees of Freedom	Allantoinase Activity	
		MV	H_2O_2
Pre-Conditioning	2	3.89*	0.94
Treatment	1	64.01***	81.01***
Pre-Conditioning*Treatment	2	0.80	0.35

Source of Variation	Degrees of Freedom	Percent of Control	
		MV	H_2O_2
Pre-Conditioning	2	1.05	0.02

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-16: Specific allantoinase activity in isolated leaf disks during and induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Allantoinase Activity	
		MV	H_2O_2
Pre-Conditioning	2	5.36**	5.12**
Treatment	1	29.62***	39.01***
Pre-Conditioning*Treatment	2	1.65	0.01

Source of Variation	Degrees of Freedom	Percent of Control	
		MV	H_2O_2
Pre-Conditioning	2	1.96	0.40

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-17: Protein content in isolated leaf disks during and induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Allantoinase Activity	
		MV	H_2O_2
Pre-Conditioning	2	0.22	0.20
Treatment	1	33.62***	41.62***
Pre-Conditioning*Treatment	2	1.29	0.06

Source of Variation	Degrees of Freedom	Percent of Control	
		MV	H_2O_2
Pre-Conditioning	2	0.06	0.11

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-18: Lipid peroxidation in isolated leaf disks during and induced oxidatve stress treatment.

Source of Variation	Degrees of Freedom	Lipid Peroxidation	
		MV	H_2O_2
Pre-Conditioning	2	0.47	0.06
Treatment	1	2.57	4.88*
Pre-Conditioning*Treatment	2	0.03	0.18

Source of Variation	Degrees of Freedom	Percent of Control	
		MV	H_2O_2
Pre-Conditioning	2	0.08	0.96

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-19: Ureide content in tepary bean leaves treated with methyl viologen.

Source of Variation	Degrees of Freedom	Ureide Content		
		Uric Acid	Allantoin	Allantoate
Genotype	28	0.86	1.69*	3.57***
Treatment	1	0.21	1.36	0.91
Genotype*Treatment	28	0.77	1.15	0.85

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-20: Correlation between the preliminary and repeated experiment assessing tepary beans response to induced oxidative stress.

	Uric Acid Control Ex.1	Allantoin Control Ex.1	Allantoate Control Ex.1	Uric Acid MV Ex. 1	Allantoin MV Ex. 1	Allantoate MV Ex.1	Uric Acid Control Ex.2	Allantoin Control Ex.2	Allantoate Control Ex.2	Uric Acid MV Ex. 2	Allantoin MV Ex. 2	Allantoate MV Ex.2
Uric Acid Control Ex.1	r=1	r=-0.002 p=0.991	r=0.061 p=0.767	r=0.048 p=0.815	r=-0.121 p=0.557	r=0.225 p=0.270	r=-0.087 p=0.674	r=-0.030 p=0.883	r=0.104 p=0.613	r=-0.087 p=0.672	r=0.047 p=0.821	r=0.033 0.8724
Allantoin Control Ex.1		r=1	r=0.785 p<0.001	r=0.278 p=0.170	r=0.280 p=0.166	r=0.406 p=0.040	r=0.452 p=0.021	r=0.466 p=0.017	r=0.665 p<0.001	r=0.116 p=0.573	r=0.508 p=0.008	r=0.572 p=0.002
Allantoate Control Ex. 1			r=1	r=-0.058 p=0.778	r=0.459 p=0.018	r=0.759 p<.0001	r=0.067 p=0.745	r=0.012 p=0.953	r=0.095 p=0.645	r=-0.023 p=0.910	r=0.025 p=0.904	r=0.080 p=0.698
Uric Acid Control Ex.1				r=1	r=-0.240 p=0.237	r=-0.234 p=0.250	r=0.226 p=0.268	r=0.403 p=0.042	r=0.511 p=0.008	r=0.104 p=0.613	r=0.459 p=0.018	r=0.412 p=0.034
Allantoin Control Ex.1					r=1	r=0.643 p<0.001	r=-0.088 p=0.668	r=0.048 p=0.815	r=-0.078 p=0.704	r=0.016 p=0.940	r=-0.054 p=0.794	r=0.228 p=0.262
Allantoate Control Ex. 1						r=1	r=-0.197 p=0.334	r=-0.126 p=0.541	r=-0.134 p=0.516	r=-0.112 p=0.585	r=-0.143 p=0.485	r=0.002 p=0.991
Uric Acid Control Ex.2							r=1	r=0.823 p<0.001	r=0.656 p<0.001	r=0.598 p=0.001	r=0.600 p=0.001	r=0.545 p=0.004
Allantoin Control Ex.2								r=1	r=0.757 p<0.001	r=0.434 p=0.027	r=0.765 p<0.001	r=0.723 p<0.001
Allantoate Control Ex. 2									r=1	r=0.230 p=0.257	r=0.861 p<0.001	r=0.894 p<0.001
Uric Acid Control Ex.2										r=1	r=0.306 p=0.128	r=0.312 p=0.121
Allantoin Control Ex.2											r=1	r=0.896 p<0.001
Allantoate Control Ex. 2												1

No statistical correlations were significant.

Table A-21: Comparison of the percent change in superoxide content, hydrogen peroxide content and electrolyte leakage in leaf disks after treatment in chilling bath.

Source of Variation	Degrees of Freedom		
		Instant	24 Hours
Treatment- O_2^-	16	0.62	0.96
Treatment- H_2O_2	16	1.09	2.23*
Treatment- EL %	16	3.04***	

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-22: Electrolyte leakage in leaf disks after treatment in chilling bath.

Source of Variation	Degrees of Freedom	Electrolyte Leakage
Genotype	16	0.28
Treatment	1	242.69***
Genotype*Treatment	16	0.24

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-23: Correlations between superoxide content, hydrogen peroxide content and electrolyte leakage in leaf disks chilled in a cold bath.

	Instant H_2O_2	Instant O_2^-	H_2O_2 After 24	O_2^- After 24	EL Percent	EL Difference
Instant H_2O_2	1	r= -0.290 p= 0.046	r= 0.354 p= 0.014	r= -0.017 p= 0.908	r= 0.003 p= 0.983	r= -0.346 p= 0.016
Instant O_2^-		1	r= -0.185 p= 0.209	r= -0.121 p= 0.413	r= 0.078 p= 0.596	r= 0.472 p= <0.001
H_2O_2 After 24			1	r= -0.243 p= 0.097	r= -0.175 p= 0.235	r= -0.290 p= 0.0458
O_2^- After 24				1	r= 0.290 p= 0.0458	r= 0.281 p= 0.099
EL Percent					1	r= 0.506 p= <0.001
EL Difference						1

Table A-24: Percent survival of a population after sub-zero temperature stress in 2013-2015.

Source of Variation	DF	2013	2014a	2014b		2015	
		One Day	Seven Days	One Day	Seven Days	One Day	Seven Days
Genotype	134	0.95	3.55^{***}	3.30^{***}	1.92^{***}	1.22	1.59^{***}

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-25: Uric acid, allantoin and allantoate content in a sub-zero temperature stress trial in 2014 and 2015.

Source of Variation	DF	2013			2014		
		Uric Acid	Allantoin	Allantoate	Uric Acid	Allantoin	Allantoate
Day	2	11.37^{***}	2.62^{***}	3.72^{***}	5.58^{***}	3.23^{***}	2.46^{***}
Treatment	46	24.46^{***}	37.09^{***}	17.85^{***}	12.32^{***}	23.09^{***}	40.64^{***}
Day*Treatment	46	1.03	1.66^{***}	1.42^{*}	0.97	1.22	1.16

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). Analysis of 2014 allantoin, and 2013 uric acid and allantoate was completed on the square root of the original results. DF: Degrees of Freedom.

Table A-26: Correlation between ureide content and survival after sub-zero temperature stress in the 2013 trial.

	Allantoin Before Stress	Uric Acid Before Stress	Allantoate Before Stress	Allantoin During Stress	Uric Acid During Stress	Allantoate During Stress	Allantoin After Stress	Uric Acid After Stress	Allantoate After Stress	Percent of Plants Alive
Allantoin Before Stress	r=1 df=170	r=0.165 p=0.031 df=170	r=0.868 p<.001 df=170	r=0.138 p=0.094 df=148	r=0.155 p=0.060 df=148	r=0.116 p=0.150 df=150	r=0.178 p=0.030 df=150	r=0.113 p=0.167 df=151	r=0.111 p=0.168 df=151	r=0.028 p=0.717 df=170
Uric Acid Before Stress		r=1 df=170	r=0.055 p=0.485 df=170	r=-0.083 p=0.314 df=148	r=0.319 p<.001 df=148	r=-0.155 p=0.05 df=150	r=-0.175 p=0.03 df=150	r=0.485 p<.001 df=151	r=-0.242 p=0.002 df=151	r=0.040 p=0.600 df=170
Allantoate Before Stress			r=1 df=171	r=0.213 p=0.010 df=148	r=-0.021 p=0.797 df=148	r=0.266 p=0.001 df=150	r=0.351 p=<.001 df=150	r=-0.132 p=0.106 df=151	r=0.276 p<0.001 df=151	r=0.003 p=0.972 df=171
Allantoin During Stress				r=1 df=161	r=0.007 p=0.934 df=160	r=0.914 p<.001 df=161	r=0.166 p=0.044 df=148	r=-0.100 p=0.226 df=149	r=0.217 p=0.008 df=149	r=-0.018 p=0.820 df=161
Uric Acid During Stress					r=1 df=161	r=-0.032 p=0.690 df=161	r=-0.213 p=0.009 df=148	r=0.651 p<.001 df=149	r=-0.266 p=0.001 df=149	r=0.080 p=0.314 df=161
Allantoate During Stress						r=1 r=163	r=0.231 p=0.005 df=149	r=-0.195 p=0.017 df=150	r=0.350 p<.001 df=150	r=-0.002 p=0.815 df=163
Allantoin After Stress							r=1 df=164	r=-0.162 p=0.038 df=164	r=0.746 p<.001 df=164	r=-0.075 p=0.338 df=164
Uric Acid After Stress								r=1 df=165	r=-0.217 p<0.001 df=165	r=0.180 p=0.021 df=165
Allantoate After Stress									r=1 df=165	r=0.057 p=0.456 df=165
Percent of Plants Alive										r=1 df=187

Table A-27: Correlation between ureide content and survival after sub-zero temperature stress in the 2014 trial.

	Allantoin Before Stress	Uric Acid Before Stress	Allantoate Before Stress	Allantoin During Stress	Uric Acid During Stress	Allantoate During Stress	Allantoin After Stress	Uric Acid After Stress	Allantoate After Stress	Difference in Uric Acid	Percent of Plants Alive
Allantoin Before Stress	r=1 df=162	r=0.208 p=0.008 df=162	r=0.864 p<.001 df=162	r=0.257 p=0.002 df=149	r=-0.027 p=0.745 df=149	r=0.238 p=0.004 df=149	r=0.260 p=0.001 df=161	r=0.116 p=0.144 df=161	r=0.302 p<0.001 df=161	r=0.098 p=0.218 df=161	r=0.039 p=0.625 df=157
Uric Acid Before Stress		r=1 df=162	r=0.146 p=0.064 df=162	r=0.033 p=0.687 df=149	r=0.246 p=0.003 df=149	r=-0.022 p=0.790 df=149	r=0.135 p=0.089 df=161	r=0.180 p=0.023 df=161	r=0.136 p=0.087 df=161	r=0.088 p=0.269 df=161	r=0.390 p<.001 df=157
Allantoate Before Stress			r=1 df=162	r=0.221 p=0.007 df=149	r=-0.140 p=0.090 df=149	r=0.246 p=0.003 df=149	r=0.215 p=0.006 df=161	r=0.027 p=0.735 df=161	r=0.295 p<0.001 df=161	r=0.014 p=0.856 df=161	r=-0.067 p=0.406 df=157
Allantoin During Stress				r=1 df=158	r=0.370 p<.001 df=158	r=0.961 p<.001 df=158	r=0.121 p=0.133 df=156	r=0.143 p=0.074 df=156	r=0.113 p=0.162 df=156	r=0.108 p=0.189 df=149	r=0.033 p=0.688 df=152
Uric Acid During Stress					r=1 df=158	r=0.256 p=0.001 df=158	r=0.039 p=0.631 df=156	r=0.418 p<.001 df=156	r=-0.026 p=0.748 df=156	r=0.252 p=0.002 df=149	r=0.447 p<.001 df=152
Allantoate During Stress						r=1 df=158	r=0.110 p=0.174 df=156	r=0.044 p=0.584 df=156	r=0.125 p=0.121 df=156	r=0.050 p=0.543 df=149	r=-0.037 p=0.642 df=152
Allantoin After Stress							r=1 df=173	r=0.642 p<.001 df=173	r=0.848 p<.001 df=173	r=0.690 p<.001 df=162	r=0.020 p=0.794 df=166
Uric Acid After Stress								r=1 df=173	r=0.485 p<.001 df=173	r=0.996 p<.001 df=162	r=0.198 p=0.011 df=166
Allantoate After Stress									r=1 df=173	r=0.495 p<.001 df=162	r=-0.048 p=0.542 df=166
Difference in Uric Acid										r=1 df=162	r=0.168 p=0.035 df=157
Percent of Plants Alive											r=1 df=169

Table A-28: Yield, biomass and harvest index in a water limitation trial in 2013.

Source of Variation	DF	Individual Genotypes			Groups		
		Yield	Biomass	Harvest Index	Yield	Biomass	Harvest Index
Genotype/Group	123/2	4.98***	1.93***	7.22***	19.24***	9.14***	8.45***
Treatment	1	389.97***	602.83***	0.68	6.83**	42.51***	4.85*
Genotype/ Group *Treatment	123/2	1.49**	1.12	1.43**	10.32***	1.22	6.17**

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-29: Ureide content in leaves in water limitation stress trial.

Source of Variation	2013		
	Uric Acid	Allantoin	Allantoate
Trial	0.63	6.08**	4.84**
Trial*Genotype	0.57	2.12**	0.87
Trial*Treatment	0.59	10.74***	3.96*
Trial*Genotype *Treatment	0.67	2.36**	0.87

A repeated measured ANOVA was used to determine if differences between genotypes exist. Numbers in table indicate the F Value and asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***). Degrees of freedom are 3 for the trials, 42 for the genotypes and 1 for the treatment. Adjustments used Greenhouse-Geisser-Epsilon adjustment.

Table A-30: Drought scores during water limitation trial in 2013.

Source of Variation	DF	Individual Genotypes			Groups		
		Day 6	Day 8	Day 10	Day 6	Day 8	Day 10
Genotype	125/2	3.24***	3.48***	3.54***	13.11***	28.88***	38.59***

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***). DF: Degrees of Freedom.

Table A-31: Correlations between ureides and agronomic traits in the water limitation trial in 2013.

	BM	YLD	HI	Score 6	Score 8	Score 10	UA 4	UA 6	UA 8	UA 10	ALN 4	ALN 6	ALN 8	ALN 10	ALT 4	ALT 6	ALT 8	ALT 10
BM	r=1 df=64	r= 0.611 p<.001 df=63	r=-0.192 p=0.132 df=63	r=-0.355 p=0.046 df=32	r=-0.521 p=0.002 df=32	r=-0.282 p=0.118 df=32	r=-0.411 p=0.003 df=50	r=0.324 p=0.011 df=61	r=0.095 p=0.464 df=62	r=-0.112 p=0.389 df=61	r=-0.216 p=0.133 df=50	r=-0.151 p=0.242 df=62	r=0.246 p=0.054 df=62	r=0.086 p=0.508 df=61	r=-0.094 p=0.512 df=50	r=-0.190 p=0.139 df=62	r=0.201 p=0.123 df=60	r=0.032 p=0.807 df=61
YLD		r=1 df=63	r=0.609 p<.001 df=63	r=-0.242 p=0.181 df=32	r=-0.295 p=0.101 df=32	r=-0.141 p=0.440 df=32	r=-0.360 p=0.010 df=50	r=0.195 p=0.135 df=60	r=0.158 p=0.225 df=61	r=-0.123 p=0.350 df=60	r=-0.004 p=0.976 df=50	r=-0.216 p=0.095 df=61	r=-0.031 p=0.810 df=61	r=0.091 p=0.490 df=60	r=0.222 p=0.117 df=50	r=-0.012 p=0.930 df=61	r=0.022 p=0.866 df=59	r=0.134 p=0.307 df=60
HI			r=1 df=63	r=0.010 p=0.955 df=32	r=0.076 p=0.680 df=32	r=0.078 p=0.670 df=32	r=-0.077 p=0.597 df=50	r=-0.063 p=0.631 df=60	r=0.069 p=0.600 df=61	r=-0.068 p=0.608 df=60	r=0.182 p=0.205 df=50	r=-0.074 p=0.572 df=61	r=-0.156 p=0.231 df=61	r=-0.032 p=0.811 df=60	r=0.302 p=0.031 df=50	r=0.187 p=0.148 df=61	r=-0.054 p=0.686 df=59	r=0.094 p=0.475 df=60
Score 6				r=1 df=32	r=0.858 p<.001 df=32	r=0.833 p<.001 df=32	r=-0.139 p=0.527 df=23	r=-0.154 p=0.426 df=29	r=0.073 p=0.694 df=31	r=0.230 p=0.213 df=31	r=-0.204 p=0.350 df=23	r=-0.215 p=0.255 df=30	r=-0.291 p=0.112 df=31	r=-0.337 p=0.064 df=31	r=0.035 p=0.871 df=23	r=0.081 p=0.671 df=30	r=-0.227 p=0.228 df=30	r=-0.143 p=0.444 df=31
Score 8					r=1 df=32	r=0.792 p<.001 df=32	r=-0.151 p=0.491 df=23	r=-0.275 p=0.149 df=29	r=-0.004 p=0.982 df=31	r=0.107 p=0.566 df=31	r=-0.108 p=0.623 df=23	r=-0.274 p=0.143 df=30	r=-0.181 p=0.329 df=31	r=-0.269 p=0.143 df=31	r=0.041 p=0.949 df=23	r=-0.137 p=0.472 df=30	r=-0.137 p=0.470 df=30	r=-0.097 p=0.602 df=31
Score 10						r=1 df=32	r=-0.387 p=0.068 df=23	r=-0.264 p=0.166 df=29	r=-0.164 p=0.378 df=31	r=0.247 p=0.180 df=31	r=-0.138 p=0.530 df=23	r=-0.092 p=0.630 df=30	r=-0.006 p=0.976 df=31	r=-0.256 p=0.165 df=31	r=0.223 p=0.294 df=23	r=0.197 p=0.296 df=30	r=0.037 p=0.848 df=30	r=-0.046 p=0.807 df=31
UR 4							r=1 df=50	r=-0.175 p=0.235 df=48	r=0.226 p=0.119 df=49	r=-0.031 p=0.832 df=50	r=0.271 p=0.057 df=50	r=0.236 p=0.106 df=48	r=-0.092 p=0.531 df=49	r=0.000 p=0.997 df=50	r=-0.055 p=0.704 df=50	r=0.086 p=0.562 df=48	r=-0.073 p=0.619 df=49	r=0.083 p=0.565 df=50
UR 6								r=1 df=61	r=0.315 p=0.015 df=59	r=-0.041 p=0.757 df=58	r=-0.232 p=0.112 df=48	r=0.025 p=0.849 df=61	r=-0.118 p=0.374 df=59	r=0.099 p=0.461 df=58	r=-0.218 p=0.132 df=48	r=-0.239 p=0.064 df=61	r=-0.221 p=0.098 df=57	r=-0.071 p=0.595 df=58
UR 8									r=1 df=62	r=-0.061 p=0.645 df=59	r=0.070 p=0.635 df=49	r=-0.194 p=0.138 df=60	r=-0.138 p=0.286 df=62	r=0.022 p=0.868 df=59	r=0.093 p=0.522 df=49	r=-0.139 p=0.288 df=60	r=-0.217 p=0.096 df=60	r=0.138 p=0.298 df=59
UR 10										r=1 df=61	r=-0.077 p=0.596 df=50	r=-0.114 p=0.391 df=59	r=-0.112 p=0.398 df=59	r=0.003 p=0.985 df=61	r=0.066 p=0.645 df=50	r=0.020 p=0.880 df=59	r=-0.118 p=0.373 df=59	r=-0.017 p=0.897 df=61
ALN 4											r=1 df=50	r=0.536 p<.001 df=48	r=-0.063 p=0.668 df=49	r=0.122 p=0.400 df=50	r=0.730 p<.001 df=50	r=0.459 p<.001 df=48	r=0.111 p=0.448 df=49	r=0.165 p=0.253 df=50
ALN 6												r=1 df=62	r=0.366 p<.001 df=60	r=0.070 p=0.596 df=59	r=0.41 p=0.003 df=48	r=0.624 p<.001 df=62	r=0.370 p=0.004 df=58	r=0.238 p=0.070 df=59
ALN 8													r=1 df=62	r=0.107 p=0.421 df=59	r=-0.145 p=0.315 df=49	r=0.119 p=0.364 df=60	r=0.923 p<.001 df=60	r=0.156 p=0.239 df=59
ALN 10														r=1 df=61	r=0.239 p=0.091 df=50	r=0.081 p=0.541 df=59	r=0.115 p=0.384 df=59	r=0.535 p<.001 df=61
ALT 4															r=1 df=50	r=0.678 p<.001 df=48	r=0.038 p=0.796 df=49	r=0.298 p=0.033 df=50
ALT 6																r=1 df=62	r=0.210 p=0.114 df=58	r=0.256 p=0.050 df=59
ALT 8																	r=1 df=60	r=0.293 p=0.024 df=59
ALT 10																		r=1 df=61

Table A-32: Correlation between difference in ureide content and difference in agronomic traits in a water limitation trial in 2013.

	BM Diff	YLD Diff	HI Diff	UA4 Diff	UA6 Diff	UA8 Diff	UA10 Diff	ALN4 Diff	ALN6 Diff	ALN8 Diff	ALN10 Diff	ALT4 Diff	ALT6 Diff	ALT8 Diff	ALT10 Diff
BM Diff	r=1 df=16	r=0.414 p=0.111 df=16	r=-0.102 p=0.707 df=16	r=0.151 p=0.577 df=16	r=0.268 p=0.315 df=16	r=-0.087 p=0.749 df=16	r=-0.075 p=0.783 df=16	r=0.483 p=0.058 df=16	r=0.572 p=0.021 df=16	r=0.152 p=0.574 df=16	r=0.305 p=0.252 df=16	r=0.315 p=0.235 df=16	r=0.572 p=0.021 df=16	r=-0.011 p=0.969 df=15	r=0.005 p=0.985 df=16
YLD Diff		r=1 df=16	r=0.695 p=0.003 df=16	r=-0.214 p=0.427 df=16	r=-0.043 p=0.874 df=16	r=-0.033 p=0.905 df=16	r=0.321 p=0.226 df=16	r=0.357 p=0.175 df=16	r=0.045 p=0.868 df=16	r=-0.125 p=0.644 df=16	r=0.115 p=0.671 df=16	r=0.518 p=0.040 df=16	r=0.455 p=0.077 df=16	r=-0.233 p=0.403 df=15	r=-0.131 p=0.628 df=16
HI Diff			r=1 df=16	r=-0.470 p=0.066 df=16	r=-0.069 p=0.800 df=16	r=-0.046 p=0.865 df=16	r=0.403 p=0.122 df=16	r=-0.008 p=0.977 df=16	r=-0.362 p=0.168 df=16	r=-0.198 p=0.462 df=16	r=-0.203 p=0.450 df=16	r=0.236 p=0.380 df=16	r=0.278 p=0.297 df=16	r=-0.216 p=0.438 df=15	r=-0.134 p=0.628 df=16
UA4 Diff				r=1 df=16	r=0.097 p=0.721 df=16	r=0.101 p=0.710 df=16	r=-0.278 p=0.298 df=16	r=0.149 p=0.581 df=16	r=0.422 p=0.103 df=16	r=-0.077 p=0.776 df=16	r=0.421 p=0.104 df=16	r=-0.009 p=0.973 df=16	r=-0.037 p=0.891 df=16	r=-0.228 p=0.414 df=15	r=-0.061 p=0.823 df=16
UA6 Diff					r=1 df=16	r=0.350 p=0.185 df=16	r=0.056 p=0.836 df=16	r=-0.105 p=0.699 df=16	r=0.214 p=0.426 df=16	r=0.111 p=0.684 df=16	r=-0.044 p=0.870 df=16	r=-0.305 p=0.251 df=16	r=-0.179 p=0.507 df=16	r=-0.016 p=0.955 df=15	r=-0.207 p=0.442 df=16
UA8 Diff						r=1 df=16	r=0.108 p=0.691 df=16	r=0.096 p=0.723 df=16	r=-0.161 p=0.551 df=16	r=-0.097 p=0.722 df=16	r=-0.367 p=0.161 df=16	r=0.027 p=0.920 df=16	r=-0.054 p=0.843 df=16	r=-0.216 p=0.439 df=15	r=-0.637 p=0.008 df=16
UA10 Diff							r=1 df=16	r=-0.169 p=0.531 df=16	r=-0.347 p=0.188 df=16	r=-0.205 p=0.445 df=16	r=-0.036 p=0.894 df=16	r=0.204 p=0.448 df=16	r=0.027 p=0.921 df=16	r=-0.174 p=0.536 df=15	r=0.002 p=1 df=16
ALN4 Diff								r=1 df=16	r=0.577 p=0.019 df=16	r=-0.385 p=0.141 df=16	r=-0.011 p=0.967 df=16	r=0.790 p<.001 df=16	r=0.672 p=0.004 df=16	r=-0.276 p=0.319 df=15	r=0.089 p=0.744 df=16
ALN6 Diff									r=1 df=16	r=0.109 p=0.688 df=16	r=0.356 p=0.177 df=16	r=0.188 p=0.485 df=16	r=0.240 p=0.371 df=16	r=0.062 p=0.827 df=15	r=0.436 p=0.091 df=16
ALN8 Diff										r=1 df=16	r=0.097 p=0.721 df=16	r=-0.548 p=0.028 df=16	r=-0.179 p=0.508 df=16	r=0.879 p<.001 df=15	r=0.062 p=0.820 df=16
ALN10 Diff											r=1 df=16	r=0.089 p=0.743 df=16	r=0.069 p=0.800 df=16	r=-0.293 p=0.290 df=15	r=0.393 p=0.132 df=16
ALT4 Diff												r=1 df=16	r=0.635 p=0.008 df=16	r=-0.518 p=0.048 df=15	r=0.022 p=0.936 df=16
ALT6 Diff													r=1 df=16	r=-0.241 p=0.386 df=15	r=-0.032 p=0.907 df=16
ALT8 Diff														r=1 df=15	r=0.162 p=0.565 df=15
ALT10 Diff															r=1 df=16

Table A-33: Yield and hundred-seed weight in a water limitation trial.

Source of Variation	Degrees of Freedom	Individual Genotypes		Groups	
		Yield	HSW	Yield	HSW
Plant	96/2	6.02***	18.36***	158.90***	136.97***
Treatment	1	58.91***	109.36***	27.64***	5.23*
Plant*Treatment	96/2	1.33*	0.72	13.00***	0.46

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-34: Ureide content in leaves of tepary bean, common bean and interspecific introgression lines during the water limitation trial in 2014.

Source of Variation	Degrees of Freedom	Individual Genotypes			Groups		
		Uric Acid	Allantoin	Allantoate	Uric Acid	Allantoin	Allantoate
Plant	44/2	3.34***	2.47***	2.30***	4.13*	9.67***	15.19***
Treatment	1	3.32	25.31***	38.71***	0.09	3.07	4.26*
Plant*Treatment	44/2	0.86	1.43	1.09	0.40	0.65	1.60

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-35: Drought scores in water limitation trial in 2014.

Source of Variation	Degrees of Freedom	Individual Genotypes	Groups
		Scores	Scores
Genotype	96/2	5.26***	40.89***

Asterisks indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Table A-36: Correlations in 2014 water limitation stress trial.

	HSW	Yield	Drought Score	Uric Acid	Allantoin	Allantoate
HSW	r=1 df=573	r=-0.253 p<.001 df=566	r=0.324 p<.001 df=287	r=0.101 p=0.104 df=258	r=0.236 p<.001 df=258	r=0.260 p<.001 df=258
Yield		r=1 df=574	r=-0.434 p<.001 df=287	r=-0.176 p=0.004 df=262	r=-0.239 p<.001 df=262	r=-0.278 p<.001 df=262
Drought Score			r=1 df=291	r=0.125 p=0.150 p=134	r=0.178 p=0.040 p=135	r=0.236 p=0.006 p=134
Uric Acid				r=1 df=268	r=-0.225 p<.001 p=268	r=-0.251 p<.001 p=268
Allantoin					r=1 df=268	r=0.898 p<.001 p=268
Allantoate						r=1 p=268

Table A-37: Correlations between differences of treated and control in 2014 water limitation stress trial.

	HSW Difference	Yield Difference	Drought Score	Uric Acid Difference	Allantoin Difference	Allantoate Difference
HSW Difference	r=1 df=97	r=0.005 p=0.958 df=97	r=0.120 p=0.241 df=97	r=-0.053 p=0.730 df=45	r=0.0311 p=0.840 df=45	r=0.104 p=0.495 df=45
Yield Difference		r=1 df=97	r=0.267 p=0.008 df=97	r=-0.081 p=0.599 df=45	r=-0.058 p=0.703 df=45	r=-0.089 p=0.562 df=45
Drought Score			r=1 df=97	r=-0.277 p=0.065 df=45	r=0.144 p=0.345 df=45	r=0.344 p=0.021 df=45
Uric Acid Difference				r=1 df=45	r=-0.493 p<.001 df=45	r=-0.562 p<.001 df=46
Allantoin Difference					r=1 df=45	r=0.884 p<.001 df=45
Allantoate Difference						r=1 df=45

Table A-38: Yeild and drought scores from the water limitation trial in 2015.

Source of Variation	Degrees of Freedom	2015	
		Yield	Drought Score
Genotype	94	9.01^{***}	4.96^{***}
Treatment	1	98.27^{***}	
Genotype*Treatment	94	0.48	

Asterisk indicate significance (p-value: <0.05*, <0.01**, <0.001***).

Appendix B- Leaf disk experiment supplementary material

Additional information and results of the isolated leaf disk experiment are reported in this appendix. Data include the percent of changes of values determined by analysis between the treatment and control, the allantoinase activity on a leaf disk basis (in contrast to the specific activity reported in the results), and the full data sets of ureide content in both the leaves and the solution the leaves were floated in. The appendix also contains the large tepary survey ureide content results.

Table B-1: Percent of control of treated leaves in the isolated leaf disk experiment.

Treatment	Pre-Conditioning	Superoxide (O_2^-)	Hydrogen Peroxide (H_2O_2)	Electrolyte Leakage	Allantoinase Activity	Protein	Lipid Peroxides
		(%)					
H_2O_2	**Water**	106 [a]	226 [a]	347 [a]	73 [a]	67 [a]	132 [a]
	Allantoin	103 [a]	261 [a]	208 [b]	77 [a]	66 [a]	188 [a]
	Uric Acid	105 [a]	196 [a]	258 [ab]	71 [a]	70 [a]	137 [a]
MV	**Water**	107 [a]	127 [a]	311 [a]	80 [a]	76 [a]	129 [a]
	Allantoin	110 [a]	82 [a]	192 [b]	74 [a]	78 [a]	148 [a]
	Uric Acid	105 [a]	77 [a]	304 [ab]	63 [a]	73 [a]	135 [a]

Lower case letters indicate differences within the treatment.

Table B-2: Allantoinase activity on a leaf disk basis in the isolated leaf disk experiment.

		Pre-Conditioning		
		Water	**Allantoin**	**Uric Acid**
		nkat/leaf disk		
Treatment	**Water**	0.064 ± 0.005^{Aa}	0.073 ± 0.005^{Aa}	0.066 ± 0.004^{Aa}
	MV	0.036 ± 0.005^{Bab}	0.046 ± 0.004^{Ba}	0.028 ± 0.004^{Bb}
	H_2O_2	0.032 ± 0.006^{Ba}	0.034 ± 0.003^{Ba}	0.033 ± 0.004^{Ba}

At least five replicates were used for each value. Upper case letters indicate differences within the pre-conditioning solutions and lower case letters indicate differences within the treatment.

Table B-3: Ureide content in the leaf in the isolated leaf disk experiment.

Ureide	Pre-conditioning		ROS treatment	
	nmol/leaf disk ± standard error			
Uric Acid	**Water**	0.000 ± 0.000	**Control**	0.380 ± 0.240
			H_2O_2	0.734 ± 0.099
			MV	0.380 ± 0.246
	Allantoin	0.000 ± 0.000	**Control**	0.188 ± 0.162
			H_2O_2	1.718 ± 0.305
			MV	0.481 ± 0.326
	Uric Acid	20.126 ± 3.747	**Control**	33.852 ± 4.570
			H_2O_2	7.778 ± 5.401
			MV	20.440 ± 3.272
Allantoin	**Water**	7.806 ± 1.087	**Control**	9.420 ± 0.877
			H_2O_2	6.565 ± 0.616
			MV	3.028 ± 0.718
	Allantoin	23.882 ± 1.429	**Control**	40.554±5.280
			H_2O_2	18.824 ± 1.307
			MV	13.085 ± 1.445
	Uric Acid	10.458 ± 1.102	**Control**	10.851 ± 0.451
			H_2O_2	8.172 ± 0.601
			MV	3.152 ± 0.443
Allantoate	**Water**	0.000 ± 0.000	**Control**	0.000 ± 0.000
			H_2O_2	0.000 ± 0.000
			MV	0.759 ± 0.357
	Allantoin	43.689 ± 2.83	**Control**	98.855 ± 11.515
			H_2O_2	35.625 ± 9.012
			MV	30.959 ± 6.878
	Uric Acid	0.000 ± 0.000	**Control**	0.000 ± 0.000
			H_2O_2	0.000 ± 0.000
			MV	0.000 ± 0.000

A minimum of three replicates were used for each value.

Table B-4: Ureide concentration of the supernatant in the isolated leaf disk experiment.

Ureide	Pre-Conditioning			ROS Treatment		
		With Leaf	No Leaf		With Leaf	No Leaf
	uM ± standard error					
Uric Acid	Water	0	0	Control	1 ± 0	0
				H_2O_2	2 ± 0	0
				MV	1 ± 0	0
	Allantoin	0	0	Control	0	0
				H_2O_2	0	0
				MV	1 ± 0	0
	Uric Acid	991 ± 43	1047 ± 33	Control	744 ± 65	1045 ± 23
				H_2O_2	739 ± 48	903 ± 49
				MV	919 ± 59	1056 ± 21
Allantoin	Water	0	0	Control	0	0
				H_2O_2	1 ± 1	0
				MV	0	0
	Allantoin	862 ± 9	1040 ± 46	Control	309 ± 54	1011 ± 36
				H_2O_2	825 ± 15	1046 ± 54
				MV	736 ± 29	967 ± 25
	Uric Acid	7 ± 7	0	Control	23 ±11	1 ± 0
				H_2O_2	62 ± 11	14 ± 7
				MV	49 ± 23	0
Allantoate	Water	0	0	Control	0	0
				H_2O_2	0	0
				MV	0	0
	Allantoin	154 ± 7	0	Control	164 ± 31	0
				H_2O_2	251 ± 10	0
				MV	380 ± 21	0
	Uric Acid	0	0	Control	8 ± 5	0
				H_2O_2	11 ± 6	0
				MV	18 ± 12	0

A minimum of three replicates were used for each value.

Table B-5: Ureide content in leaves of the large tepary experiment.

	Uric Acid		Allantoin		Allantoate	
	MV	Control	MV	Control	MV	Control
Cultivar	**nmol/leaf disk**					
Tepary 19	0.101	0.183	4.692	6.404	57.139	85.361
G40144	0.14	0.068	0	0.139	3.35	8.469
G40152	0.043	0.049	0.062	1.193	0.118	0.146
G40142A	0.479	0.766	0	0	0	0
G40111	0.124	0.333	0	0	0.12	0
G40172	0.111	0.1	0.093	0.112	0.155	0.226
Tepary Gold	0.098	0.162	0	0	0	0
G40175	0.09	0.144	0.073	0.066	0	0
G40158A	0.058	0.064	0	0	0	0
G40030	0.134	0.134	0	0	0	0
G40038	0	0	0	0	0	0
G40141	0	0	0	0	0	0
G40166	0	0	0	0	0	0
G40283	0	0	0	0	0	0
G40165	0.05	0.043	0	0	0	0
G40067	0.056	0.047	0	0	0	0
G4011	0.046	0.036	0	0	0	0
G40060	0.087	0.064	0	0	0	0
G40034	0.388	0.343	0.133	0.1	0.548	0.599
G40028	0.397	0.036	0.527	1.592	1.129	0.368
G40127	0.126	0	0.061	0.112	0	0
Tepary 29	0.158	0.081	0	0	0	0
G40023	0.23	0.127	0	0.033	0.109	0.098
G40302	0.239	0.152	0	0	0	0
G40040	0.087	0	0	0	0	0
G40300	0.128	0.04	0	0	0	0
G40110	0.691	0.737	0	0	0.141	0
Tepary 23	0.137	0.106	0.071	0	0	0
G40058	0.469	0.35	0.08	0.064	0	0
G40201	0.069	0.072	0	0	0.168	0
G40151	0.767	0.594	0	0	0	0
G40173C	0.119	0.116	0	0	0.174	0
G40125	0.07	0	0.079	0.093	0.135	0
G40138	0.381	0.16	0	0	0	0
G40157	0.188	0.095	0	0	0.133	0
G40174	0.302	0.069	0	0	0	0
Tepary1	0.409	0.159	0	0	0	0
G40035	0.323	0.071	0	0	0	0
G40022	0.752	0.093	0	0.617	0.236	0

continued

Continued Table B-5: Ureide content in leaves of the large tepary experiment.

	Uric Acid		Allantoin		Allantoate	
	MV	Control	MV	Control	MV	Control
Cultivar	**nmol/leaf disk**					
Tepary White	0.105	0.052	0	0	0.232	0
Mitla Black	0.203	0.037	0	0	0.12	0
G40119	0.186	0.053	0	0	0.246	0.08
G40144A	0.956	0.65	0	0	0	0
G40020	0.056	0.077	0	0	0.333	0
G40057	0.081	0.063	0.094	0	0.214	0
G40176	0.601	0.274	0	0	0	0
G40154	0.55	0.22	0	0	0	0
G40128	0.338	0.123	0	0	0.119	0
G40237A	0.551	0.126	0.065	0.087	0.173	0.234
Tepary Gray	0.1	0.078	0	0	0.471	0.146
G40159	0	0	0	0	0.349	0
G40301	0.394	0.04	0	0	0	0
G40135	1.107	0.745	0	0	0	0
G40130	1.176	0.813	0	0	0	0
G40149	0.755	0.365	0	0	0	0
G40145	0.289	0	0	0	0.12	0
G40143	0.479	0.044	0	0	0	0
G40150	0.708	0.269	0	0	0	0
G40147	0.555	0.115	0	0	0	0
G40144C	0.21	0.07	0	0	0.306	0
G40161	0.34	0.43	0.569	0.597	0.848	0.247
G40022A	0.088	0.912	0	0	1.566	0.233
G40068	1.076	0.525	0	0	0	0
Tepary 32	0.322	0.077	0	0	0.332	0
G40012	0.092	0	0.067	0	0.443	0
G40164	0.066	0.121	0	0	0.842	0.128
G40140	1.025	0.304	0	0	0	0
G40156	0.413	0.178	0	0	0.545	0
G40162	0.225	0	0	0	0.56	0
G4006B	0.493	0.188	0.172	0	0.335	0
Tepary 4	1.176	0.328	0	0	0	0
G4008	0.285	0.164	0.062	0.073	1.157	0.402
G40160	0.228	0.161	0.062	0	0.939	0.186
G40122	1.029	0.346	0	0	0.214	0
G40061	0.158	0.069	0.284	0.042	0.575	0
G40142	1.052	0.05	0	0	0	0

continued

Continued Table B-5: Ureide content in leaves of the large tepary experiment.

	Uric Acid		Allantoin		Allantoate	
	MV	Control	MV	Control	MV	Control
Cultivar	**nmol/leaf disk**					
G40129	0.336	0.074	0.145	0	0.597	0
G40010	0.879	0.18	0.116	0.081	0.505	0.17
G40041	0.879	0.18	0.116	0.081	0.505	0.17
G40042	0.284	0.165	0	0	1.062	0
G4177A1	1.174	0.421	0.089	0	0.403	0
T40177B1	1.479	0.655	0.069	0	0.465	0
Tepary 22	1.549	0.389	0	0	0.229	0
G40146	0.585	0.102	0.799	0.159	3.299	2.907
G40043	2.376	0.444	0	0.345	0.12	0.121
G40016	0.506	0.473	0.296	0.193	4.552	3.091
G40839	0.131	0.186	0.175	0.138	3.914	2.296
G40025	0.162	0.166	0.06	0	2.338	0.607
Tepary 3	2.441	0.494	0	0	0	0
G40120	0.486	0.074	0.134	0	1.681	0.065
G40299	0.182	0.129	0.087	0	4.101	0.881
G40037	0.267	0.099	0.128	0	3.068	0
G40008	0.405	0.541	0.706	0.069	3.365	0.301
G40013A	0.675	0.422	0.479	0.073	3.373	0.239
G40066A	0.519	0.135	0.121	0.069	3.994	0.137
G40009	3.833	0.038	0	0	0.731	0
G40001	0.185	0.157	1.009	0.158	5.937	1.917
Tepary 6	1.177	0.087	0.305	0.065	5.338	1.71
G40037A	0.614	1.732	2.764	1.498	27.641	20.725
G40148	7.961	0.277	0	0	0	0
G40021	1.273	0.071	1.044	0.051	9.436	1.2
G40173	0.962	0.701	2.158	0.843	29.16	20.142
G40036	0.593	0.101	0.221	0.207	11.957	0
G40018	0.125	0.113	1.546	0	45.758	2.683
Tepary 2	0.256	0.047	4.82	0.124	47.477	4.868
G40029	0.173	0.284	13.14	5.687	79.879	36.986
G40032	0.383	0.271	3.734	1.126	71.457	18.435
G40144B	0.235	0.106	0	0	0.15	0

Appendix C- Temperature data for field trials

The temperature data for the field experiments was recorded either in field (sub-zero temperature trial) or by a nearby weather station (water limitation trial). A summary of these temperatures is included in the following tables for both field trials.

Table C-1: Air temperatures (°C) and precipitation during the water limitation trials in Isabela, Puerto Rico as well as the historical averages (1971-2000) at the Isabela Substation (18.465°N 67.052°W).

		Average Maximum (°C)	Average Minimum (°C)	Precipitation (inches)
2013	**January**	27.7	20.3	2.15
	February	28.1	19.8	1.46
	March	28.3	19.9	3.98
	April	29	21	3.65
2014	**January**	28.2	19.8	0.37[A]
	February	29.8 [B]	18.5 [B]	1.07 [A]
	March	29	19.6	1.84
	April	30.7 [B]	18.6 [B]	0.33 [A]
2015	**January**	28.2	20.2	2.6 [A]
	February	28	20.5	2.6
	March	28.1	19.9	2.15
	April	29.7	20.7	2.22
Historical Average	**January**	27.6	18.6	3.01
	February	27.7	18.4	3.27
	March	28.3	18.7	3.38
	April	29	19.4	4.79

Where recording data was missing, values were collected from nearby stations, including [A] Mora Camp, PR (18.474°N 67.029°W) and [B] Coloso, PR (18.381°N 67.157°W).

Table C-2: Air temperatures (°C) during sub-zero temperature trial and the historical average temperatures for the same time period.

Date		2013		2014		2015		Historical	
		High	Low	High	Low	High	Low	High	Low
August	18							24.9	12.3
	19								
	20								
	21								
	22								
	23								
	24								
	25					27.8	11.8		
	26					29.0	7.6		
	27					31.4	9.9		
	28					31.7	11.5		
	29					33.5	17.0		
	30					29.9	16.5		
	31					30.1	12.0		
September	1					30.7	8.9	18.4	6.4
	2			15.3		31.4	12.1		
	3			19.7	6.8	20.8	12.0		
	4			24.6	6.6	18.7	9.7		
	5			24.6	8.3	12.1	9.1		
	6			27.9	10.4	13.0	10.2		
	7			32.7	10.2	14.0	7.3		
	8			9.9	6.7	19.6	5.3		
	9			8.6	2.7	20.7	0.9		
	10			9.8	1.9	24.3	1.4		
	11			15.5	2.9	28.1	5.9		
	12			18.7	-1.5	32.8	9.2		
	13			15.5	5.9	22.9	10.7		
	14			20.4	-2.7	17.7	7.7		
	15	30.2		17.5	2.5	11.6	9.0		
	16	28.9	13.0	29.2	1.8	13.9	8.4		
	17	17.7	7.2	22.2	7.0	17.8	7.3		
	18	19.4	8.1	27.8	8.5	23.6	1.2		
	19	24.1	0.0	24.1	9.7	26.7	2.7		
	20	28.3	6.9	22.5	8.5	26.3	6.4		
	21	23.4	10.6	30.1	2.2	19.2	7.7		
	22	25.9	7.6	32.7	7.6	19.7	-2.0		
	23	26.1	4.2	34.2	9.5	22.5	6.8		
	24	10.1	8.6	30.2	6.6	24.6	6.0		
	25	9.4	7.8	31.2	10.9	32.0	10.2		
	26	17.4	3.4	29.0	11.1	23.9	6.4		
	27	20.2	2.3	12.0	6.8	20.9	3.6		
	28	23.1	7.0	13.0	1.9	15.7	-2.7		
	29	19.1	2.2	18.3	-0.3	25.0	0.7		
	30	12.4	6.8	15.8	10.4	28.6	8.3		
October	1	9.9	0.2	17.7	7.9			10.5	-0.1
	2	11.3	-0.5	5.8	1.4				
	3	16.1	-4.6	8.4	-3.5				
	4	16.0	1.1	10.6	-0.8				
	5	18.8	6.1	12.8	-1.0				
	6	21.9	2.9	11.4	3.2				
	7	21.7	2.3	15.3	2.8				
	8	28.9	3.1	13.0	-4.0				
	9	23.0	4.0	17.5	-3.3				
	10			22.3	1.9				

Green-filled boxes indicate the sowing date of the planting that was used in the analysis. Blue-filled boxes indicate the first detrimental frost event. Two plantings were analyzed in 2014 as the planting sown on Sept. 2, 2014 avoided the first frost and was analyzed during the second frost.

Appendix D- Field trials ureide content

Ureides were measured in the leaves of several genotypes in both field trials. The below data are the numerical values reported in graphical form in section 3.9.1 and 3.9.2. Correlations of the quantities of specific ureides with each other are also represented for each experiment.

Table D-1: Ureide quantity in leaves during 2013 sub-zero temperature trial.

		Uric Acid			Allantoin			Allantoate		
		Before	Day Of	After	Before	Day Of	After	Before	Day Of	After
		nmol/mg DW								
Common Bean	**Expresso**	0.07	0.07	0.02	1.70	1.37	1.25	6.45	4.51	2.73
	NY5-161	0.05	0.02	0.06	0.66	1.62	1.95	6.14	4.52	2.9
	Pintium	0.07	0.03	0.04	3.26	1.13	2.37	14.51	2.7	2.58
Inter-specific Line	**A-10-12**	0.04	0.05	0.05	3.32	2.28	4.14	11.99	6.7	6.68
	A-11-11	0.00	0.00	0.00	0.51	1.26	6.59	3.13	4.51	9.11
	A-14-10	0.09	0.07	0.05	1.59	2.19	4.97	10.18	6.94	5.92
	A-14-6	0.13	0.06	0.07	5.64	3.24	8.82	20.21	7.43	12.29
	A-14-8	0.11	0.03	0.05	0.09	0.85	3.48	4.32	3.85	5.16
	A-3-1	0.14	0.03	0.07	0.21	2.47	5.04	3.72	5.26	7.01
	A-5-6	0.00	0.00	0.00	0.27	1.17	2.95	2.08	4	6.59
	B-11-8	0.07	0.09	0.03	0.51	1.29	2.95	4.52	4.16	3.56
	B-1-9	0.13	0.04	0.04	1.74	8.87	5.44	9.58	15.91	5.88
	B-3-1	0.09	0.05	0.05	1.19	1.44	5.49	8.18	3.57	7.23
	B-3-12	0.07	0.07	0.06	0.58	1.64	2.67	5.66	5.32	5.43
	B-6	0.05	0.05	0.04	2.37	0.90	5.55	10.23	4.49	8.7
	B-6-2	0.05	0.08	0.01	1.33	0.75	2.56	9.92	3.34	4.13
	B-6-9	0.00	0.00	0.00	0.46	0.89	2.62	3.15	5.17	5.15
	C-11-2 Pattern	0.00	0.00	0.00	1.06	1.23	2.96	5.38	4.7	5.76
	C-11-5	0.04	0.03	0.05	2.23	2.48	6.59	13.05	8.59	8.26
	D-13-4Br	0.19	0.07	0.07	2.07	0.53	8.32	8.95	1.94	10
	D-6-12	0.00	0.00	0.00	0.43	2.38	6.86	2.53	4.1	7.1
	D-7-2Br	0.00	0.00	0.00	0.73	1.14	3.57	4.85	3.56	4.36
	D-9-2Br	0.03	0.04	0.07	2.73	2.08	10.59	13.05	5.48	5.43
	D-9-3	0.00	0.00	0.00	1.17	2.14	10.07	5.45	5.97	15.56
	E-12-11	0.11	0.09	0.05	1.19	1.76	1.97	5.23	3.22	2.9
	E-6-7	0.12	0.04	0.03	1.77	1.04	2.74	9.34	4.5	4.37
	E-7-10	0.08	0.03	0.07	0.65	0.80	4.49	4.68	2.92	5.74
	F-10-3	0.00	0.00	0.00	0.67	0.62	2.81	3.59	4.14	5.1

Continued on following page

Continued Table D-1: Ureide quantity in leaves during 2013 sub-zero temperature trial.

		Uric Acid			Allantoin			Allantoate		
		Before	Day Of	After	Before	Day Of	After	Before	Day Of	After
		nmol/mg DW								
Tepary Bean	**G40001**	0.30	0.12	0.15	3.98	1.01	2.43	9.65	1.87	2.52
	G40010	0.38	0.13	0.25	6.79	0.68	1.67	14.16	1.55	1.48
	G40028	0.42	0.19	0.23	1.85	0.99	1.57	5.83	2.48	1.69
	G40036	0.28	0.25	0.39	2.43	0.45	1.91	4.54	0.83	2
	G40060	0.28	0.21	0.28	0.20	0.85	2.06	1.44	2.07	1.72
	G40061	0.44	0.11	0.23	0.71	0.97	4.22	2.21	1.33	3.05
	G40110	0.11	0.11	0.05	0.57	1.39	2.46	3.72	2.41	3.67
	G40119	0.54	0.18	0.31	1.41	0.32	2.73	3.75	0.58	2.79
	G40141	0.37	0.14	0.14	1.72	0.58	1.73	5.25	1.71	2.15
	G40144C	0.40	0.13	0.24	2.95	0.36	2.53	7.05	0.4	3.2
	G40150	0.59	0.30	0.99	1.20	0.22	0.73	3.49	0.78	1.36
	G40200	0.39	0.26	0.19	7.61	1.13	4.00	17.68	2.42	6.49
	G40201	0.23	0.17	0.24	0.23	2.57	1.53	2.05	3.44	1.68
	Tepary Gold	0.40	0.08	0.16	4.67	0.30	1.01	8.04	1.38	1.57
	Tepary White	0.00	0.02	0.04	0.53	0.00	0.41	1.41	0.45	3.01
	Tepary Gray	0.00	0.03	0.04	0.15	0.06	1.51	2.48	0.06	0.93
Unknown	**Mitla Black**	0.14	0.04	0.05	1.05	3.46	0.76	4.84	6.51	1.29

Values represent the average of four replicate.

Table D-2: Ureide quantity in leaves during 2014 sub-zero temperature trial.

		Uric Acid			Allantoin			Allantoate		
		Before	Day Of	After	Before	Day Of	After	Before	Day Of	After
		nmol/mg DW								
Common Bean	**Expresso**	0.21	0.21	0.39	0.44	0.99	9.05	3.78	5.81	30.76
	NY5-161	0.32	0.34	0.38	0.63	1.58	1.71	6.47	6.94	9.85
	Pintium	0.26	0.26	0.43	0.84	0.97	5.36	5.43	4.64	15.99
Interspecific Introgression Lines	**A-10-12**	0.23	0.20	0.19	0.65	0.71	1.42	2.94	7.08	7.43
	A-11-11	0.27	0.26	0.34	0.06	0.42	1.91	4.12	4.93	8.44
	A-14-10	0.23	0.43	0.38	1.32	1.65	6.17	10.01	6.55	15.3
	A-14-6	0.22	0.25	0.18	1.84	0.81	1.84	6.54	7.21	8.31
	A-14-8	0.24	0.30	0.25	2.23	0.63	1.96	6.65	6.69	10.93
	A-3-1	0.22	0.23	0.32	3.63	1.08	4.11	10.49	6.92	15.99
	A-5-6	0.24	0.31	0.34	3.12	0.61	1.56	7.97	4.33	7.07
	B-11-8	0.30	0.35	0.23	0.39	1.55	2.99	6.01	6.81	9.37
	B-1-9	0.36	0.43	0.28	0.01	0.77	1.41	0.91	5.06	5.49
	B-3-1	0.32	0.29	0.33	0.32	3.46	2.12	4.1	12.98	5.95
	B-3-12	0.20	0.32	0.3	1.89	1.04	1.62	9	7.83	7.8
	B-6	0.20	0.27	0.26	0.87	1.65	1.30	3.41	10.03	4.77
	B-6-2	0.16	0.25	0.25	1.30	0.27	1.17	4.26	4.9	6.27
	B-6-9	0.21	0.26	0.22	2.67	1.44	3.18	7.92	10.38	11.39
	C-11-2 Pattern	0.32	0.34	0.37	0.26	1.91	4.75	4.35	9.82	15.2
	C-11-5	0.26	0.40	0.35	1.77	1.48	1.17	5.76	9.75	5.58
	D-13-4Br	0.27	0.51	0.29	3.11	15.05	4.28	12.99	51.73	13.67
	D-6-12	0.22	0.26	0.24	2.75	1.67	3.67	7.12	9.91	13.12
	D-7-2Br	0.25	0.33	0.3	0.70	1.33	2.15	4.61	10.52	9.11
	D-9-2Br	0.25	0.26	0.29	1.59	1.82	2.67	10.14	8.33	9.99
	D-9-3	0.24	0.39	0.24	1.77	1.67	3.91	10.03	11.25	18.2
	E-12-11	0.33	0.39	0.47	0.45	0.44	1.57	3.34	4.08	7.6
	E-6-7	0.33	0.41	0.41	0.34	1.01	2.57	3.34	7.1	10.53
	E-7-10	0.34	0.27	0.17	2.20	0.71	3.38	4.83	5.25	13.24
	F- 12-7	0.26	0.36	0.23	3.48	1.58	2.05	19.28	8.25	8.86
	F-10-3	0.27	0.44	0.29	1.87	1.69	1.34	10.3	9.6	5.6
Tepary Bean	**G40001**	0.60	0.45	0.55	3.03	0.26	3.67	14.93	2.91	14.2
	G40028	0.44	0.38	0.44	0.98	0.59	1.10	2.25	4	5.43
	G40036	0.36	0.57	0.57	4.50	5.46	1.60	7.13	15.1	6.35
	G40119	0.32	0.47	0.61	0.78	3.91	2.11	0.51	11.81	6.29
	G40141	0.32	0.62	0.44	0.37	0.69	2.37	1.56	3.87	7.4
	G40144C	0.28	0.47	0.54	0.09	0.70	2.74	0.9	4.29	9.62
	G40150	0.45	0.67	0.76	5.38	2.59	3.09	12.54	8.69	10.96
	G40200	0.43	0.49	0.83	0.91	3.77	4.47	3.39	11.01	16.13
	G40201	0.78	0.57	0.45	0.33	0.30	3.47	0.93	3.98	10.07
	G40300	0.47	0.39	0.88	3.55	0.43	1.44	8.71	3.67	9.89
	Tepary Gold	0.52	0.62	0.55	2.32	3.50	2.83	7.34	12.11	8.95
	Tepary Gray	0.33	0.59	0.64	0.54	0.84	1.02	2.55	3.88	4.3
	Tepary White	0.49	0.00	0.65	1.38	0.00	0.32	4.04	0	2.36
Unknown	**Mitla Black**	0.25	0.26	0.23	0.20	0.34	0.79	3.46	6.38	4.46

Values represent the average of four replicates.

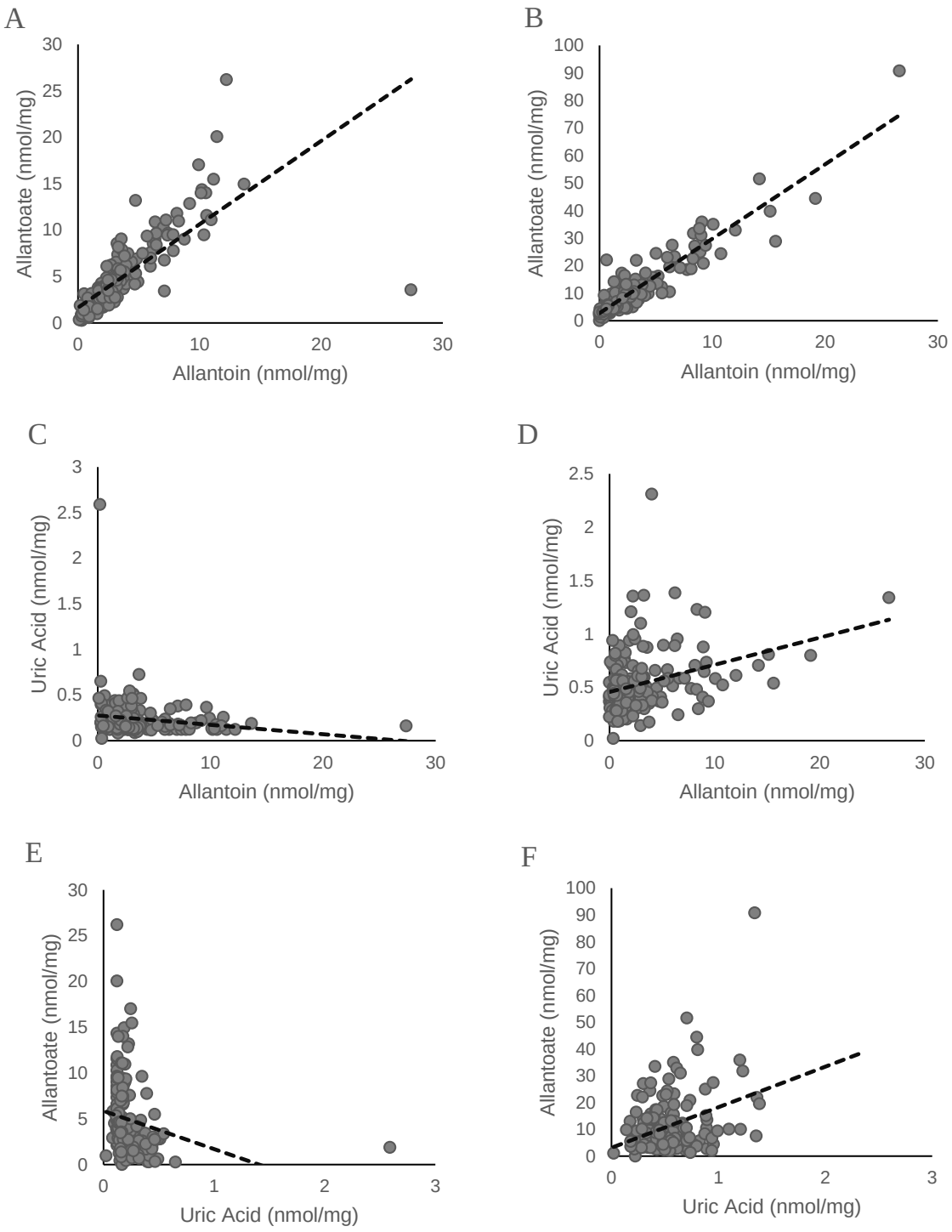

Figure D-1: Correlations between ureide content in leaves in the sub-zero temperature trial. Uric acid, allantoin and allantoate were quantified before, during and after a sub-zero temperature event in leaves of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean (shown in this figure are the ureide levels post-stress. Correlations occurred between the ureide contents (see Table A-26 and A-27 for full list). Correlations depicted were between allantoin and allantoate in 2013 (A) and 2014 (B), uric acid and allantoin in 2013 (C) and 2014 (D), and uric acid and allantoate in 2013 (E) and 2014 (F). All correlations are significant ($p<0.05$).

Table D-3: Ureide quantity in leaves during 2013 water limitation trial.

			Four		Six		Eight		Ten	
			Limited	Control	Limited	Control	Limited	Control	Limited	Control
			nmol/mg DW							
Uric Acid	Inter-specific Line	A-11-8	0.10	0.13	0.11	0.10	0.12	0.12	0.12	0.12
		A-3-4	0.23	0.11	0.12	0.13	0.12	0.12	0.13	0.13
		A-3-9	0.10	0.11	0.11	0.17	0.11	0.10	0.11	0.13
		B-11-12	0.14	0.09	0.12	0.11	0.10	0.13	0.12	0.12
		B-11-6	0.13	0.13	0.12	0.10	0.16	0.13	0.13	0.12
		B-1-8	0.13	0.12	0.11	0.14	0.12	0.13	0.40	0.12
		B-1-9	0.12	0.12	0.11	0.13	0.10	0.11	0.13	0.12
		B-5-11	0.13	0.12	0.15	0.11	0.11	0.14	0.12	0.12
		C-11-2Solid	0.12	0.11	0.10	0.14	0.12	0.10	0.12	0.10
		D-7-1Blk	0.18	0.14	0.16	0.12	0.14	0.15	0.11	0.13
		D-9-2Blk	0.13	0.12	0.10	0.12	0.08	0.10	0.12	0.11
		E-6-2	0.13	0.14	0.10	0.12	0.11	0.11	0.14	0.13
		E-7-10	0.12	0.14	0.11	0.11	0.17	0.19	0.56	0.13
		F-10-7	0.13	0.10	0.12	0.13	0.11	0.11	0.11	0.11
		F-10-8	0.14	0.11	0.10	0.12	0.12	0.10	0.10	0.15
	Common Bean	NY5-161	0.09	0.09	0.13	0.17	0.20	1.56	0.10	0.11
Allantoin	Inter-specific Line	A-11-8	2.45	0.00	1.03	0.00	2.14	1.16	0.68	0.23
		A-3-4	2.16	0.41	2.10	0.79	0.68	2.11	4.88	0.93
		A-3-9	0.84	0.11	0.82	0.63	0.98	0.82	0.97	0.75
		B-11-12	0.35	0.00	0.63	0.29	0.06	0.24	0.30	0.91
		B-11-6	0.97	0.00	0.42	0.01	0.24	1.39	0.40	0.89
		B-1-8	0.59	0.43	0.45	0.53	0.05	1.08	0.31	1.16
		B-1-9	0.60	0.10	0.83	0.48	0.08	2.69	0.02	0.29
		B-5-11	0.36	1.18	1.14	0.82	0.90	0.58	0.89	0.92
		C-11-2Solid	1.63	0.00	1.42	1.47	0.90	4.43	0.69	1.74
		D-7-1Blk	1.34	0.46	0.76	0.10	0.18	0.73	0.24	0.86
		D-9-2Blk	0.05	0.50	1.49	1.13	6.35	2.34	0.15	0.63
		E-6-2	0.09	0.05	0.85	0.37	0.28	0.17	0.56	0.99
		E-7-10	0.49	0.02	0.58	0.25	0.36	2.13	0.53	0.57
		F-10-7	0.65	1.07	1.21	0.98	0.22	0.66	0.46	0.14
		F-10-8	3.24	1.20	1.81	0.79	1.76	3.36	0.77	4.22
	Common Bean	NY5-161	0.43	0.00	1.05	0.33	0.01	0.05	2.11	0.14
Allantoate	Inter-specific Line	A-11-8	4.30	0.04	3.70	0.08	1.64	1.29	2.90	1.79
		A-3-4	4.07	0.94	4.06	1.99	0.77	3.14	5.34	4.25
		A-3-9	1.28	0.45	1.01	0.50	1.26	0.57	2.71	1.89
		B-11-12	2.15	1.33	1.84	1.27	0.16	0.43	2.14	3.99
		B-11-6	2.87	0.72	1.38	0.84	0.40	1.65	1.85	3.15
		B-1-8	2.52	1.67	2.12	1.97	0.58	1.20	1.77	3.96
		B-1-9	2.71	0.49	0.93	1.12	0.34	2.59	0.75	1.50
		B-5-11	1.86	7.43	5.04	4.57	0.95	1.24	4.38	4.68
		C-11-2Solid	5.60	0.46	5.76	1.14	0.94	3.99	1.99	7.51
		D-7-1Blk	1.67	1.89	1.31	0.82	0.18	0.91	1.44	5.48
		D-9-2Blk	0.67	2.47	4.00	2.64	5.43	3.26	0.83	3.63
		E-6-2	0.41	0.82	2.54	2.40	0.29	0.56	1.37	5.66
		E-7-10	3.83	0.46	3.16	1.19	0.72	2.10	3.76	2.49
		F-10-7	2.60	3.61	3.20	3.61	0.50	0.99	2.75	1.16
		F-10-8	7.15	5.68	4.66	3.03	4.36	3.96	5.40	3.61
	Common Bean	NY5-161	1.80	0.84	2.02	0.48			7.89	1.41

Values represent the average of two replicates.

Table D-4: Ureide quantity in leaves during 2014 water limitation trial.

		Uric Acid		Allantoin		Allantoate	
		Limited	Control	Limited	Control	Limited	Control
		nmol/mg DW					
Common Bean	**Expresso**	0.31	0.20	0.00	0.00	0.60	0.31
	NY5-161	0.15	0.29	1.69	0.61	8.51	2.67
	Pintium	0.16	0.14	0.12	0.61	1.55	4.83
Interspecific Hybrid	**A-10-12**	0.28	0.32	0.53	0.94	5.05	5.13
	A-11-11	0.15	0.18	1.54	0.58	7.95	4.15
	A-11-8	0.13	0.21	0.98	0.50	6.09	4.84
	A-14-10	0.33	0.36	0.25	0.26	2.85	1.69
	A-14-6	0.17	0.20	0.42	1.84	6.14	7.95
	A-14-8	0.19	0.23	1.12	0.34	8.70	4.08
	A-3-1	0.22	0.31	0.40	0.06	3.35	0.64
	A-3-4	0.18	0.21	1.82	1.01	5.17	5.52
	A-3-5	0.25	0.20	0.92	1.22	11.44	5.02
	A-5-6	0.13	0.19	2.52	0.51	10.09	3.24
	B-11-12	0.23	0.21	0.52	0.10	5.60	1.18
	B-11-6	0.19	0.23	1.20	0.24	7.44	2.12
	B-11-8	0.28	0.26	0.34	0.61	3.26	3.19
	B-1-8	0.26	0.31	0.77	0.45	5.40	3.09
	B-1-9	0.31	0.23	0.70	0.31	6.36	3.75
	B-3-1	0.28	0.19	0.27	1.01	2.80	6.60
	B-3-12	0.26	0.38	1.06	0.46	8.88	3.89
	B-5-11	0.19	0.15	1.36	0.75	8.25	6.72
	B-6	0.19	0.37	1.93	0.59	8.62	5.68
	B-6-9	0.14	0.17	1.69	1.11	11.05	3.97
	C-11-2Pattern	0.20	0.22	1.37	0.19	8.77	2.07
	C-11-2Solid	0.15	0.22	1.83	0.8	11.41	3.61
	C-13-6	0.22	0.21	1.02	0.29	7.80	3.13
	D-5-10	0.21	0.17	2.10	0.86	12.14	8.18
	D-6-12	0.20	0.19	2.18	1.23	12.96	5.45
	D-6-3	0.21	0.26	0.65	0.47	6.20	3.26
	D-7-2Br	0.29	0.25	0.57	0.44	4.95	3.54
	D-9-13	0.33	0.48	1.63	0.44	10.24	4.58
	D-9-2Blk	0.29	0.36	0.90	0.79	5.42	4.82
	D-9-2Br	0.21	0.25	1.60	0.76	9.01	5.96
	D-9-3	0.17	0.23	2.16	0.70	9.65	3.71
	E-10-12	0.19	0.22	0.65	0.13	5.11	1.13
	E-12-13	0.11	0.14	0.87	0.27	8.66	2.53
	E-6-7	0.16	0.15	0.62	0.95	5.30	7.28
	F-10-3	0.15	0.13	0.58	0.88	6.50	6.75
	F-10-7	0.29	0.22	0.66	0.98	5.38	5.49
	F-10-8	0.17	0.22	1.01	0.77	6.43	4.83
Tepary Bean	**G40001**	0.18	0.17	0.24	0.09	2.59	1.76
	Tepary Gold	0.21	0.23	0.25	0.35	2.21	1.96
	Tepary Gray	0.19	0.15	0.57	0.04	2.98	1.58
	Tepary White	0.12	0.11	0.17	0.10	1.78	1.01
Unknown	**Mitla Black**	0.20	0.17	0.31	0.11	2.32	1.55

Values represent the average of three replicates.

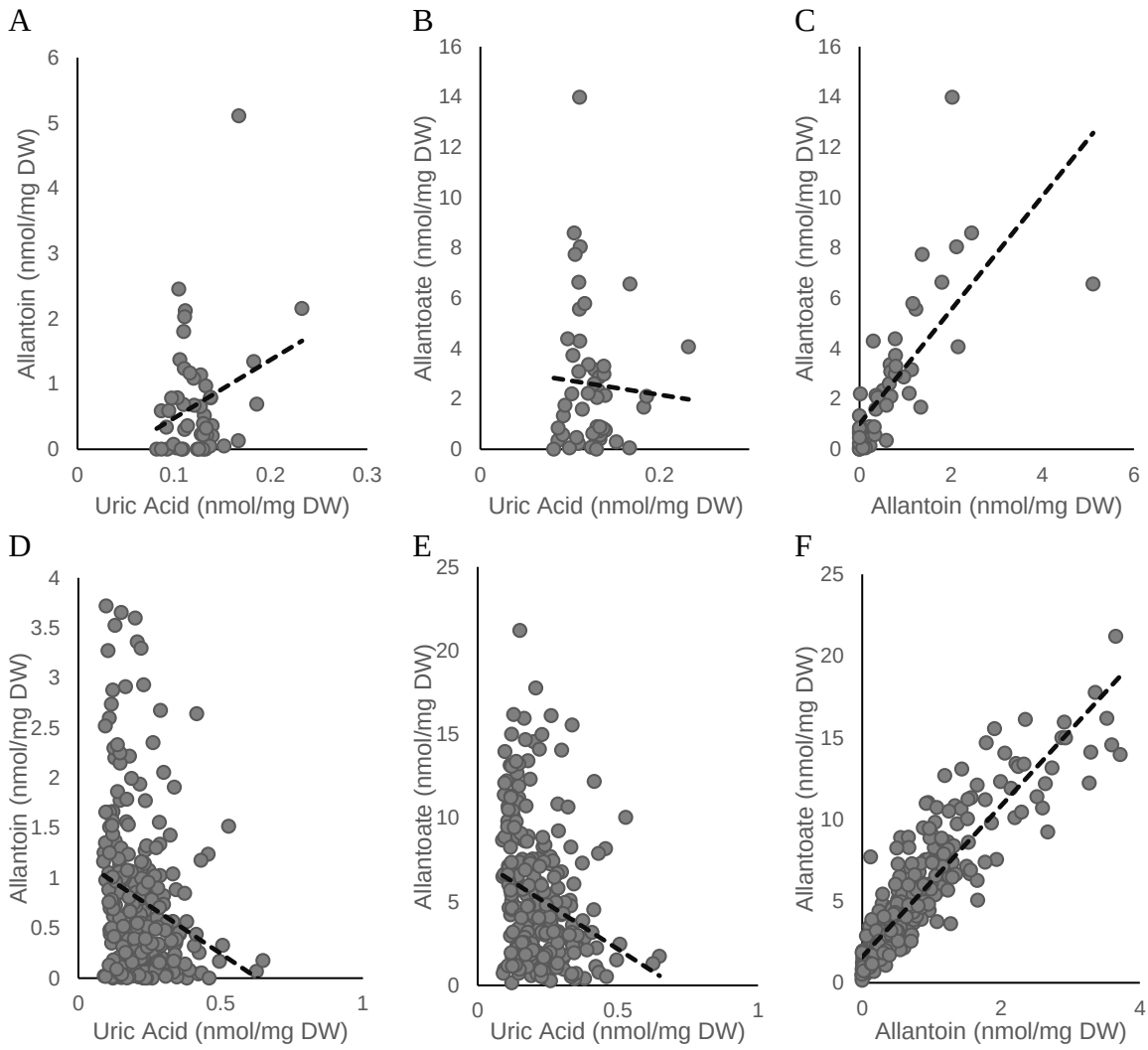

Figure D-2: Correlations between ureide content in leaves in the water limitation trial. Uric acid, allantoin and allantoate were during water limitation in leaves of common bean, tepary bean and interspecific introgression lines of common bean and tepary bean. Correlations occurred between the ureide contents (see Table A-31 and A-36 for full list). Correlations depicted were between uric acid and allantoin in (A) 2013 and (D) 2014, uric acid and allantoate in (B) 2013 and (E) 2014, and allantoin and allantoate in (C) 2013 and (F) 2014. All correlations are significant (p=0.05) except for (A) and (B).

APPENDIX E- Additional field data

Additional agronomic measurements were documented after the water limitation trial in Puerto Rico. This appendix discusses biomass and hundred-seed weight and the relationships both of these measurements have with ureide content. This appendix also includes the individual rankings of the interspecific introgression lines for survival in sub-zero temperature and two indicators of resistance to water limitation (geometric mean and tolerance).

Field trials were carried out in Puerto Rico to determine if the quantity of leaf ureides or the accumulation of ureides during stress could indicate the water limitation stress tolerance of a genotype in a population of tepary bean, common beans and interspecific introgression lines. Yield was the primary determinant of water limitation stress (discussed in section 4.4), but other agronomic characteristics were also documented to assess whether they could be used as a measure of stress tolerance and whether they had any relationship with leaf ureides (methods in 2.2.4.1). Above ground biomass (BM) was one of the additional characteristics measured in 2013 and hundred-seed weight (HSW) was calculated in the 2014 trial.

The biomass of tepary beans was greater under both irrigated and stress conditions, with interspecific introgression lines ranging in BM around the common bean checks (Figure E-1). Almost all of the genotypes, with the exception of D-5-1, had an average decrease in BM, but to varying extents. The effect of genotype and treatment were significant for BM measurements (Table A-28), but the interaction was not significant.

The intention of measuring BM was to determine if any correlations with it and ureide content existed. Biomass was negatively correlated with uric acid content on day 4 (r=-0.411), but was positively correlated with uric acid on day 6 and allantoin on day 8 (r=0.324and r=0.246, respectively). Correlations also existed between the difference of treated genotypes and control genotypes in BM and the same difference in ureide content. The difference in BM was correlated with the difference in allantoin on day 6 and the different in allantoate on day 6 (r=0.572 and r=0.560, respectively) (Figure E-2).

As plants were flowering during the initiation of the stress, hundred-seed weight (HSW) was also analyzed in 2014 to determine if genotypic differences exist due to water limitation stress. Tepary beans have a lower HSW than either the common beans or the interspecific introgression

lines. The common bean and interspecific introgression lines HSWs were not statistically different, however the average HSW of common beans (27.5g for the treated samples and 25.2g for the control samples) is larger than the average HSW of the interspecific introgression lines (24.6g for the treated samples and 22.9g for the control samples). Differences exist between genotypes and between treatments for HSW, (Figure E-3), however no interaction between stress and genotype was significant (Table A-33).

Yield and HSW were negatively correlated (r=-0.289). Interestingly, drought scores were positively correlated with HSW (r=0.324) and negatively correlated with yield (r=-0.434). Both allantoin and allantoate content positively correlated with HSW (r=0.240and r=0.266) (Figure E-4) (see Table A-36 for full correlation table).

The HSW results were unexpected as it was predicted that HSW would decrease due to the water limitation stress. However, average HSW was greater in the treated samples and the control samples. One possible reason for this occurrence could be the stress manifesting itself through flower abortion and therefore the plant had a greater ratio of resources to seeds during seed fill.

The relationships between ureides and biomass during stress and ureides and HSW should be studied further to confirm the relationships presented in this appendix.

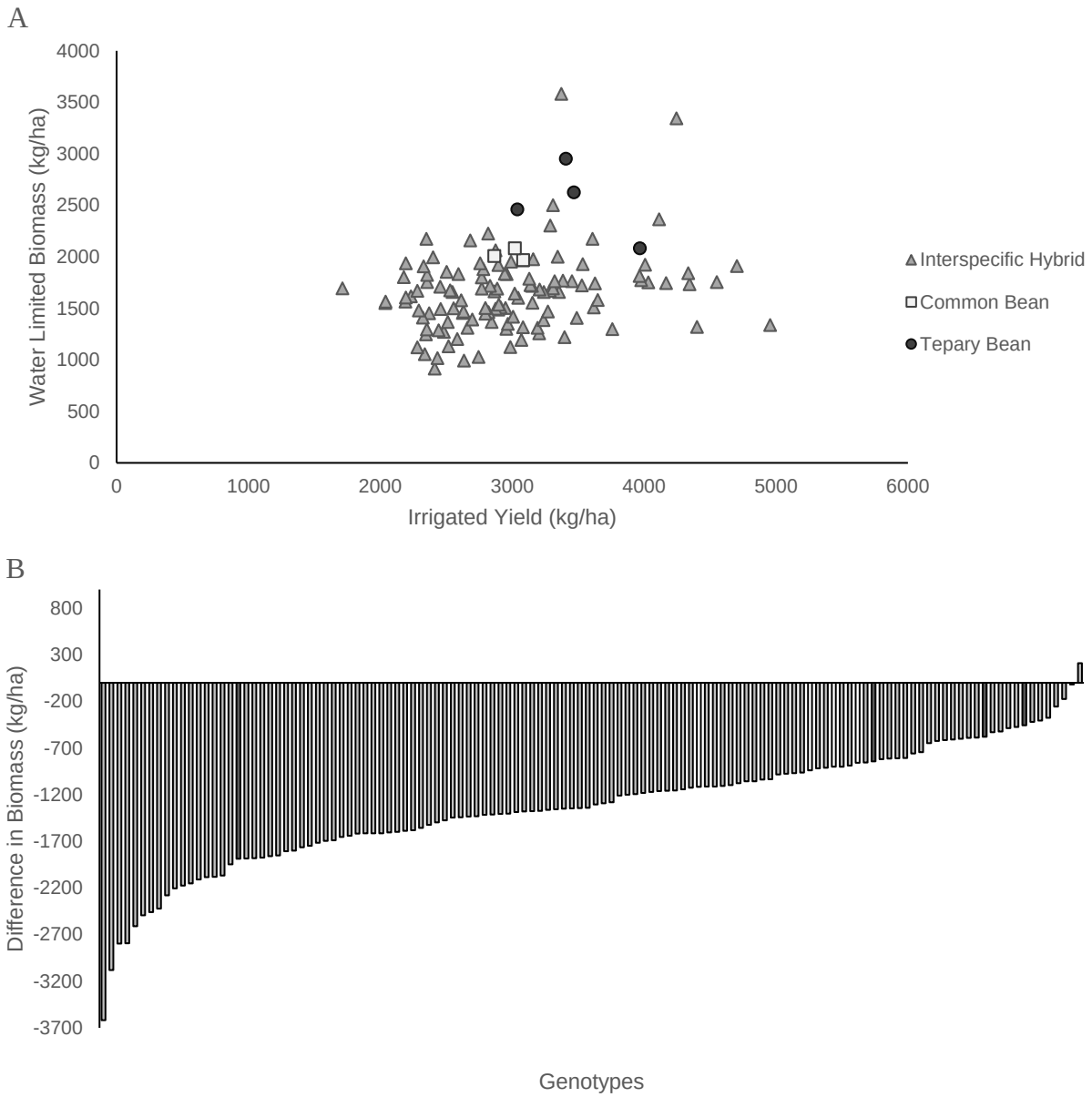

Figure E-1: Biomass of the water limitation trial in 2013 (A) Comparison of biomass of genotypes during irrigation (population average 873.39 kg/ha) and with water removed at the beginning of flowering (population average 525.44 kg/ha). (B) Difference between the average water-limited biomass and the average irrigated yield for individual genotypes. Biomass was the average of two replicates for both (A) and (B). The tepary beans are shown in black, the common bean in white and the interspecifics in gray.

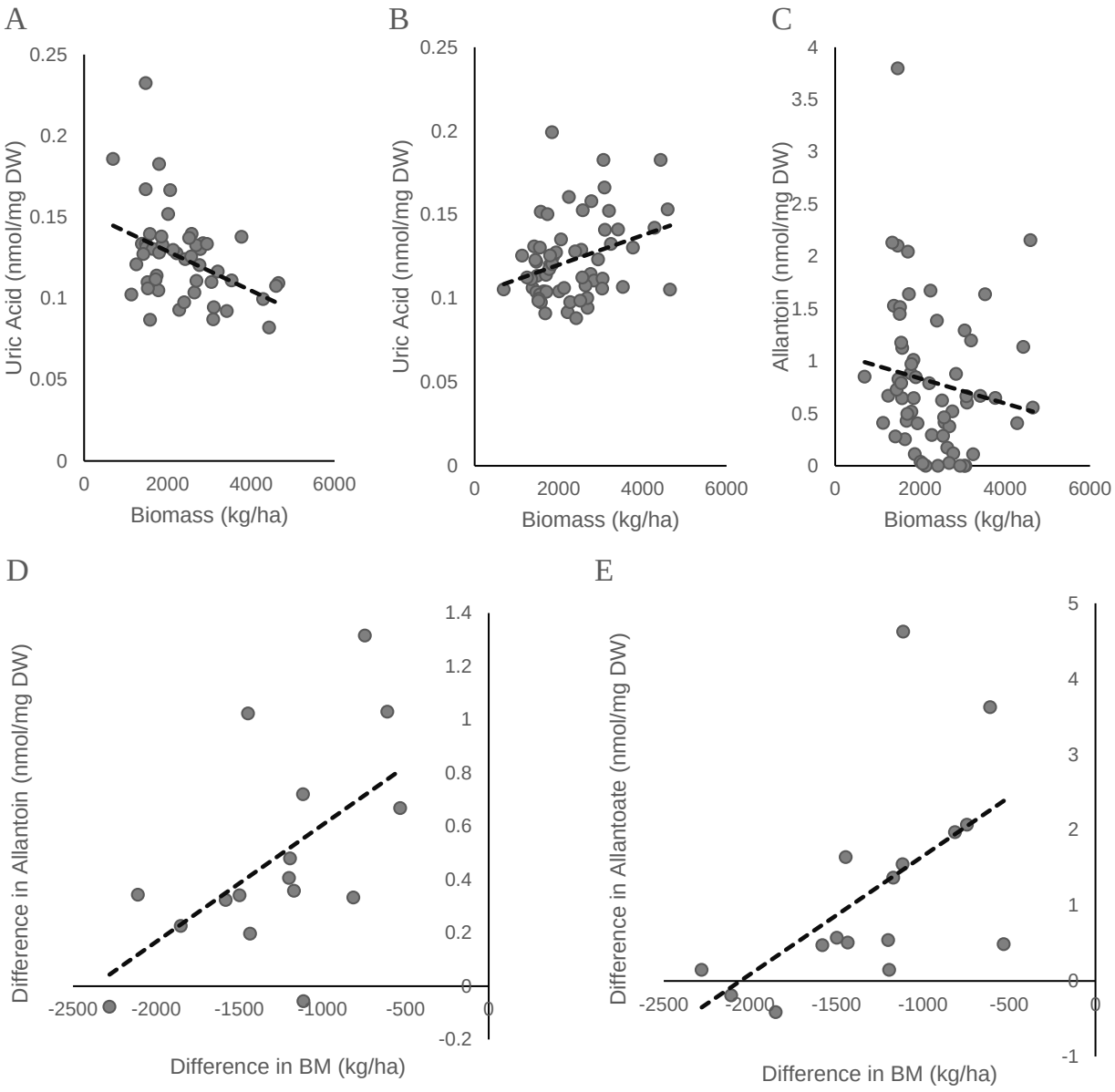

Figure E-2: Correlations with Biomass in the water limitation trial. BM was measured after the water limitation trial in 2013 and compared to the leaf content of (A) uric acid four days after water was discontinued, (B) uric acid six days after water was discontinued, and (C) allantoin six days after water was discontinued. The difference between the biomass of the water-limited plants and the control plants correlated with the difference between treated plants and control plants in (D) allantoin on day six and (E) allantoate on day six.. Full appendix table can be found in Table A-31.

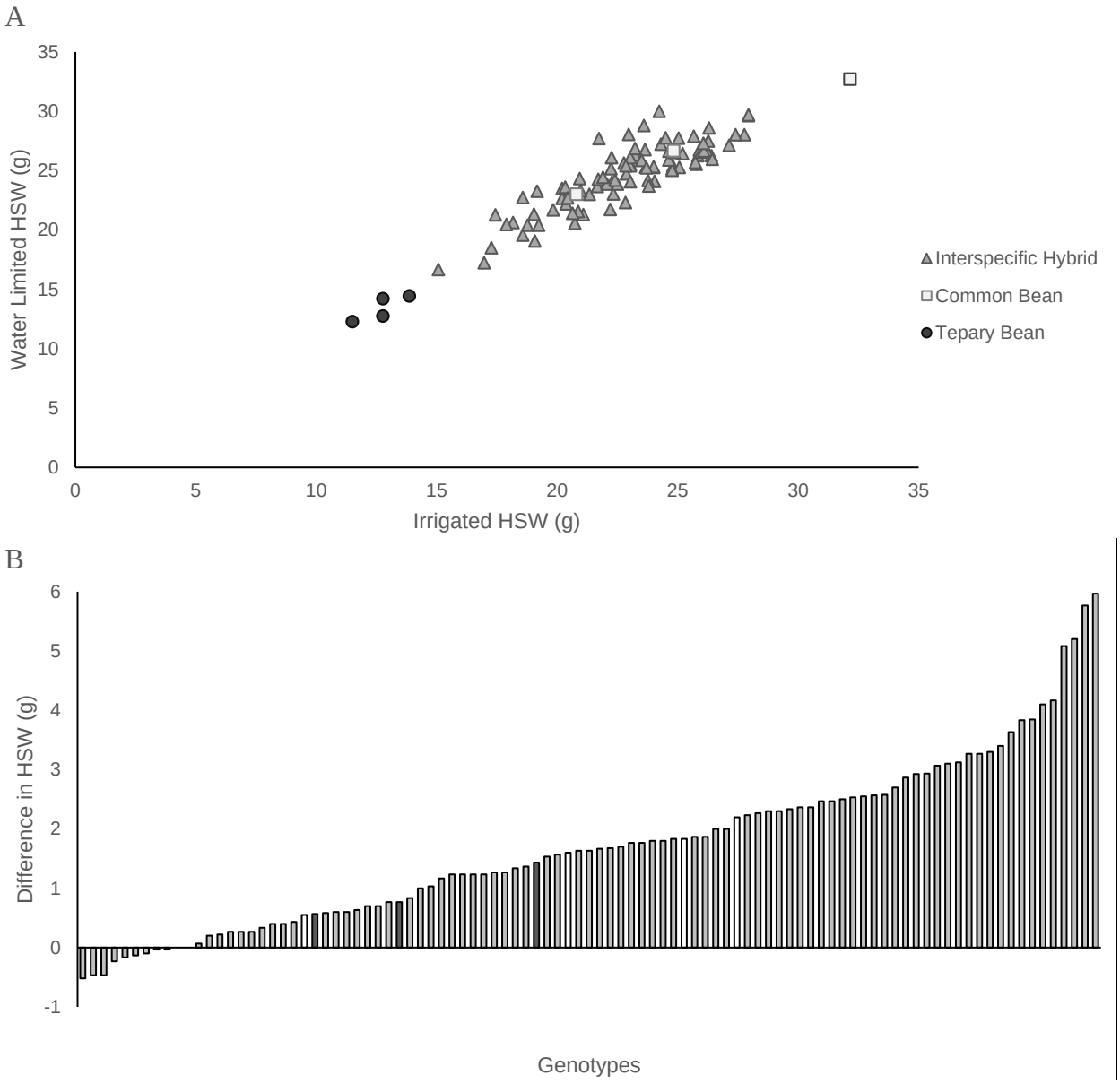

Figure E-3: Hundred-seed weight of the water limitation trial in 2014. The hundred-seed weight was measured for all accessions grown in the 2014 drought nursery. (A) A comparison on the yield and (B) the difference between the stressed treatment and the control treatment yield were calculated. The average of three replicates is shown. The tepary beans are shown in black, the common bean in white and the interspecifics in gray.

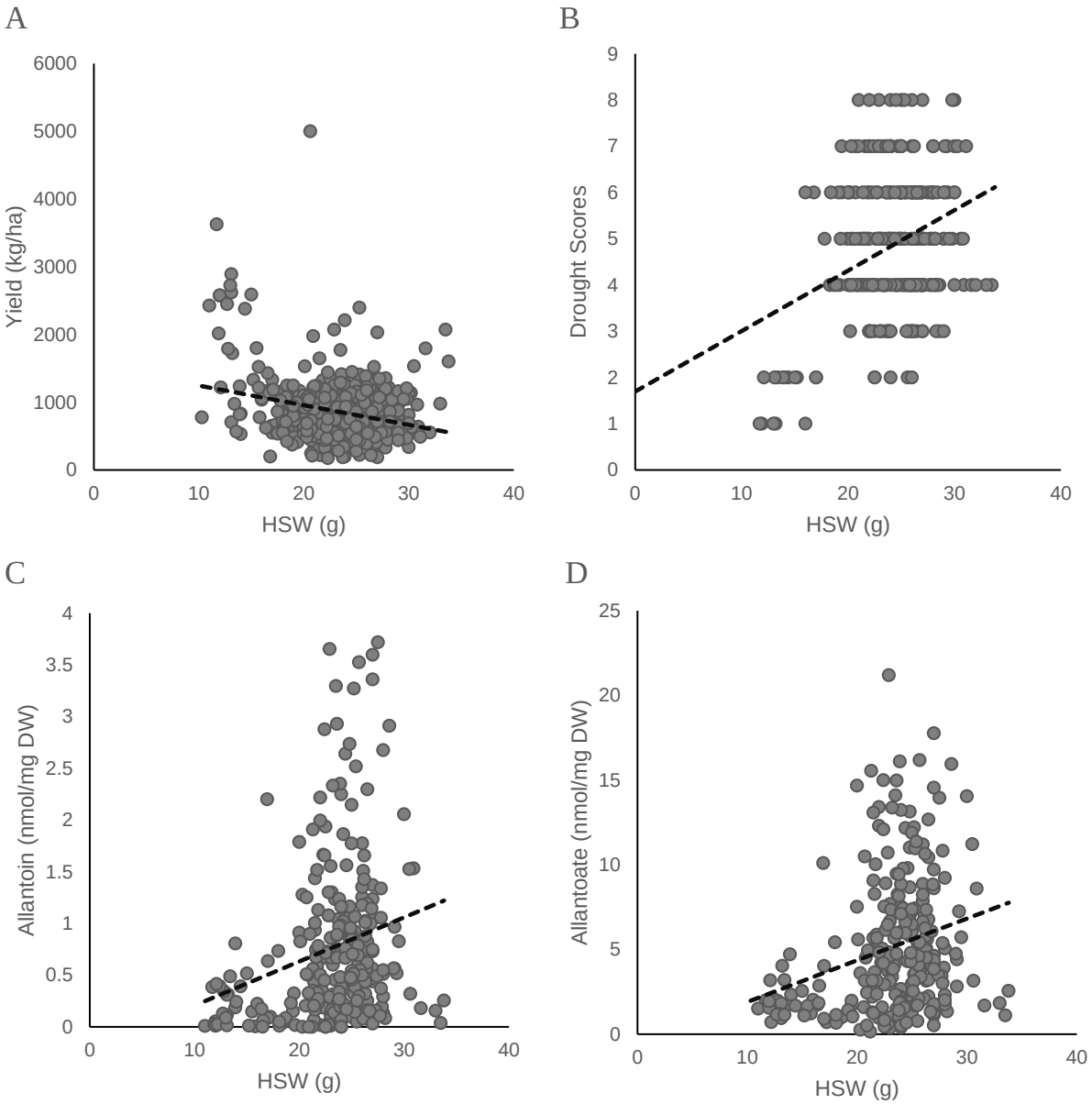

Figure E-4: Correlations with HSW in the water limitation trial. HSW was measured in 2014 after the water limitation trial and compared to the (A) yield, the (B) drought scores, (C) allantoin content in the leaves 12 days after the water was discontinued, and (D) allantoate content in the leaves 12 days after the irrigation was discontinued. Full appendix table can be found in Table A-36.

Table E-1: Promising interspecific introgression lines from sub-zero temperature and water limitation field trials.

Field Trial Ranking	Sub-Zero Temperature Trial			Water Limitation Trial					
	Survival			Geometric Mean			Tolerance		
	2014	2014b	2015	2013	2014	2015	2013	2014	2015
1	D-14-4	A-2-5-Bl	E-7-4	B-11-8	A-5-10	E-7-4	C-11-2 pattern	B-6	B-7-7 Br
2	D-11-10	E-10-11	A-3-9	F-10-3	E-6-3	B-11-3	D-1-5	B-5-5	A-3-1
3	B-3-1	D-13-4Br	D-6-6Br	D-7-2Br	D-7-2Br	C-13-6	D-5-10	B-5-11	D-5-3
4	A-3-12	B-1-13	D-9-2Blk	E-6-3	A-11-11	A-5-6	B-7-7 Br	B-6-9	E-7-4
5	D-13-4Br	B-11-12	E-6-2	B-6	A-5-5	B-11-1	C-6-5	D-14-4	B-11-1
6	A-3-13	D-6-13	C-13-6	B-6-9	F-10-12	B-2-4	E-12-8	D-9-13	A-3-12
7	E-10-8	E-6-2	D-6-12	E-9-6	F- 12-7	A-5-10	D-13-4Blk	B-7-7 Br	D-9-13
8	D-1-11	C-6-8	B-1-4	D-1-5	E-9-6	B-5-11	A-2-8	B-7-8	A-3-10
9	A-3-10	B-1-4	D-6-13	D-1-1	B-6-1	D-7-2Br	C-11-5	D-9-3	E-12-4
10	A-3-5	A-3-5	F-10-8	B-2-9	D-9-2Blk	B-11-8	B-1-4	B-7-9	F- 12-7
11	B-2-4	B-3-1	D-1-13	A-14-8	A-2-8	A-5-7	C-6-8	A-11-8	E-12-13
12	C-6-8	D-14-4	C-11-2 pattern	A-11-7	B-6-4	A-10-12	E-10-8	D-4-12	A-2-8
13	D-5-3	B-7-9	F-10-3	E-6-12	B-6-9	A-11-7	E-12-11	D-13-4Blk	B-7-8
14	D-9-3	B-1-9	A-3-10	B-6-12	B-6-5	E-6-3	D-4-12	D-7-1Br	E-9-6
15	A-3-4	B-5-5	B-6	B-5-11	E-7-5	E-7-12	A-2-5-Br	D-12-1	A-9-5
16	A-2-5-Bl	A-5-7	A-3-5	F- 12-7	B-7-7 Br	A-5-5	E-7-10	C-11-2 solid	A-14-4
17	D-9-2Br	D-7-1Br	D-7-1Br	F-10-7	A-11-7	E-6-12	E-7-4	A-14-6	D-12-1
18	E-9-6	F- 12-7	B-6-12	C-13-6	B-6	D-10-5	D-14-4	D-6-12	F-10-7
19	A-2-5-Br	A-3-1	D-1-11	B-11-6	E-12-13	D-9-2Br	D-7-1Blk	E-6-7	B-6-8
20	E-10-12	B-1-8	D-6-11Blk	A-5-7	B-5-11	A-11-11	A-10-12	A-11-11	D-13-4Blk

The twenty superior interspecific introgression lines in the field trials. The superior genotypes were determined by percent survival in the sub-zero temperature trial. The ranking in the drought trial was determined by geometric mean (relative performance under both stress and control conditions: Geometric Mean= $\sqrt{((Yi)_{Stress}*(Yi)_{Control})}$), and tolerance (difference due to stress: TOL= $(Yi)_{Control}-(Yi)_{Stress}$) calculations.

APPENDIX F- Effect on leaf storage procedure on ureide content

This appendix compares ureide content using the different leaf storage procedures that were used in this thesis.

Sampling and storage procedures differed between experiments proposed in this thesis, therefore an experiment was conducted to see what, if any, effects the different procedures would have on the ureide quantities reported. The leaves of approximately 50-55 day old nitrogen-fertilized soybean plants which had been grown in conditions previously described (see section 2.1) were sampled. Both young, fully expanded leaves and older, senescing leaves were assayed for comparison. Four different storage procedures were tested, including: leaving detached tissue at room temperature for 24 hours (day old), freezing the tissue at -80°C, oven drying the tissue, and freeze drying the tissue. Samples was then assayed by HPLC for uric acid, allantoin and allantoate content using the procedure described in section 2.3.1.

Average allantoin content was stable in the older leaves and although the frozen tissue in the younger leaves had a slightly higher average allantoin content, no difference between methods was significant (Figure F-1). Uric Acid content was stable in the young tissue. The day old leaves had lower average uric acid content, however, no difference between procedures was significant. Allantoate content was the most variable of all of the ureides. In the older tissue, although no differences were significant, the day old samples had the lowest average allantoate content and the freeze dried samples had the highest. Allantoin content was also lower in the day old samples in the young tissue, however the oven dried samples had the highest average allantoate content.

Although no differences between storage procedures were significant, determined by a one-way ANOVA, ureides in tissue left at room conditions and tissue dried or frozen may lead to differences in results and therefore may not be comparable. The different methods of drying and freezing appear to not have an effect on the ureide content of a leaf sample.

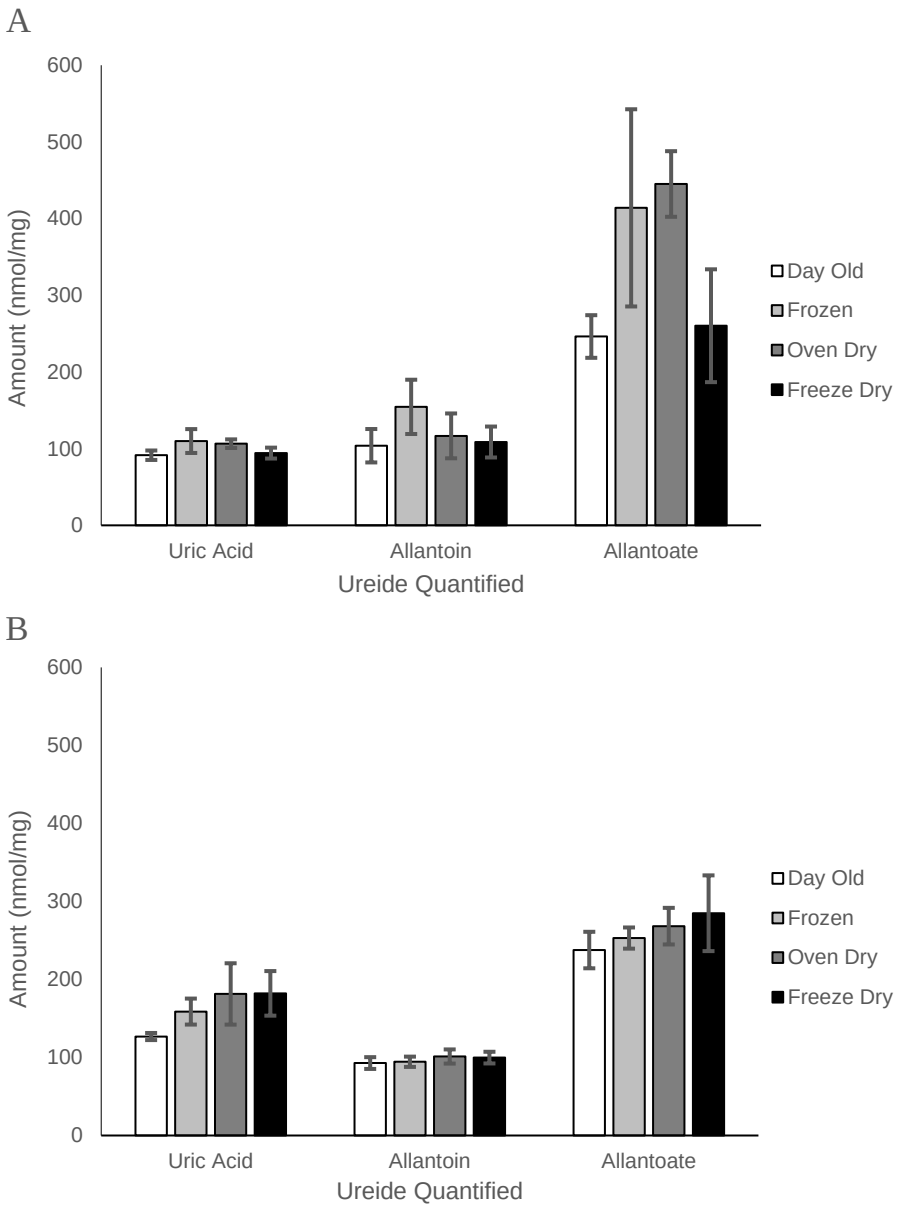

Figure F-1: Comparison of ureide content after different storage procedures. Uric acid, allantoin and allantoate were measured in nitrogen-fertilized soybean (A) younger, fully expanded and (B) older, senescing leaves. 0.2g of tissue stored in different conditions, including being: left at room temperature, frozen at -80°C, oven dried and freeze dried. Bars represent the average of three replicates ± standard error.