PHYSIOLOGICAL CHARACTERISTICS LEADING TO DIFFERENCES IN DROUGHT TOLERANCE IN *PHASEOLUS VULGARIS* AND *P. ACUTIFOLIUS*

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ABSTRACT

PHYSIOLOGICAL CHARACTERISTICS LEADING TO DIFFERENCES IN DROUGHT TOLERANCE IN *PHASEOLUS VULGARIS* AND *P. ACUTIFOLIUS*

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Common beans (*Phaseolus vulgaris* L.) are a nutritious food that provides quality protein, dietary fiber, iron, and zinc, and it is an important crop in many parts of the world, especially Central America, East Africa, and South America. Drought stress is one of the greatest limits to common bean production, for not only is drought common in areas that rely the most on beans, but many common bean cultivars in use are also sensitive to drought stress. Furthermore, under field conditions, heat stress often coincides with and exacerbates the effects of drought stress in beans. As a result, one of the major goals of bean breeding efforts is to improve drought and heat tolerance within available germplasm. To support these efforts, the research described in this dissertation examined the physiology of drought and heat stress in a selection of bean genotypes with varying degrees of stress tolerance. These genotypes included tepary bean (*Phaseolus acutifolius* A. Gray), a particularly stress tolerant species closely related to common bean. The response of different metabolites to drought stress was a major focus. Beans exposed to drought stress had no differences in free proline concentration in their leaves, either between treatments or among genotypes. For soluble carbohydrates, no differences among genotypes were found under control conditions, but the concentration of malic acid, glucose, fructose, inositol, and raffinose all increased in the leaf tissues of plants exposed to drought stress. Glucose, fructose, and inositol were all found in higher concentrations in more tolerant genotypes, so it is likely that their accumulation is correlated with drought tolerance. These compounds accumulated in sufficient quantities to osmotically adjust bean leaf tissues, and those

genotypes that accumulated more soluble carbohydrates under drought stress also had lower leaf water potentials while no differences among genotypes existed for leaf water potentials under control conditions. Abscisic acid was responsive to drought stress in beans, but differences in its concentration among genotypes did not seem directly related to drought tolerance. Grafting experiments revealed that it is shoot identity that controls the concentration of ABA in root tissues under drought stress. Drought stress also affects a number of photosynthesis related traits in beans. Photosynthesis vs. intercellular CO_2 concentration curves revealed that none of the photosynthetic parameters derived were related to drought tolerance, but the maximum carboxylation rate of rubisco and the rate of electron transport could be related to general productivity. Based on measurements of gas exchange on control and drought stressed beans, lower stomatal conductances are associated with drought tolerant genotypes regardless of water treatment. Lower stomatal conductances would allow a plant to conserve more water during periods of drought stress. Grafting experiments showed that stomatal conductance is controlled mainly by factors located in the shoot tissue and not the root tissue. However, these factors are unrelated to leaf density or the density of stomata on leaf surfaces. Bean plants exposed to temperatures of 45 °C for two days showed measurable signs of heat stress. Measures of gas exchange, chlorophyll fluorescence, and oxidative stress were for the most part only affected by this high temperature and not by any temperatures below 45 °C. These measures also correlated well with visual signs of damage on leaf tissue caused by heat stress. The method was useful for screening a large group of germplasm for heat tolerance, but this heat tolerance only partially related to drought tolerance observed in the field. Plant breeders can utilize some of the methods described in this dissertation to supplement field data and further characterize the stress tolerance of later generation bean lines.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Background information

Introduction

Common beans (*Phaseolus vulgaris* L.) are one of the world's vital crops. Nearly 400 million people, the majority of whom live in South and Central America and East Africa, depend upon common beans as their primary source of dietary protein (Broughton et al., 2003). Additionally, common beans contain high quantities of iron and zinc, two micronutrients essential to the human diet (Graham et al., 2007). Although cereal crops such as wheat, rice, and maize provide a majority of the calories consumed by humans throughout the world, legume crops such as common beans are arguably as important in the human diet for providing the protein content, essential amino acids, and nutrients that are deficient in most cereals and other starchy foods (Beebe, 2012). Furthermore, common beans are the legume crop grown in the greatest quantity for direct human consumption, its acreage grown being greater than the next two legume crops in this category combined (FAOSTAT, 2014). Improvements in the common bean germplasm available to growers could thus benefit millions of people throughout the world, and not just consumers of beans but also growers, many of whom are smallholder farmers who depend upon common beans for their livelihood (Beebe et al., 2013).

Among the many constraints to common bean production, the abiotic stressors of heat and drought are especially yield-limiting (Beebe et al., 2012; Araújo et al., 2014). Farmers are increasingly expanding bean production into marginal areas that have greater incidences of drought and soil nutrient deficiency (Beebe et al., 2013). It is estimated that more than 60% of the world's common bean production is yield-limited by drought (Cavalieri et al., 2011). Furthermore, climatologists studying the effects of climate change on agricultural production have predicted that the incidence and severity of heat stress and drought stress will increase over

the coming century (Battisti and Naylor, 2009; Lobell and Gourdji, 2012). It will be essential for plant breeders to develop new common bean varieties adapted to withstand these stresses and meet the challenges imposed by climate change. The research contained within this dissertation investigates the physiology of drought and heat stress responses within common beans and a related species with the goal that plant breeders will find this information useful when designing crosses between different varieties and evaluating the resulting lines in the field and greenhouse. Such a strategy of focusing on phenotype and stress physiology is, in conjunction with developing genetic resources and plant breeding, a key part of a strategy for common bean improvement (McClean et al., 2011). As a basis for this research, this chapter reviews the origins and characteristics of *Phaseolus* spp., its characterized responses to stress, and more general stress physiology.

Origins and genetics of common beans and tepary beans

Common beans

The nucleotide diversity at different loci sampled from several geographically distinct populations provides strong evidence for a Mesoamerican origin of common beans, arising in what is today central Mexico (Bitocchi et al., 2012). From there, common beans spread south through Central America, northern South America, and into the Andes Mountains, diverging into five distinct wild populations: Mesoamerican, Guatemalan, Colombian, Ecuadorian-northern Peruvian, and Andean (Blair, et al., 2012). Within this range of the wild species, two separate domestication events occurred, giving rise to the Andean and Mesoamerican gene pools, which are in turn subdivided into different races (Gepts and Bliss, 1985; Singh et al., 1991). While

some accessions of wild common beans are drought tolerant and well-adapted to arid environments, in the bottleneck event of domestication, much of this tolerance was lost, resulting in domesticated common beans generally being susceptible to drought stress (Cortés, et al., 2013). These wild populations could be valuable sources of abiotic stress tolerance that could be introgressed into domesticated cultivars, but as with any crossing of wild varieties into domesticated ones, plant breeders would have to avoid also introgressing any traits that negatively impact yield or quality. Within domesticated common bean germplasm today, the majority of drought resistance has come from the Mesoamerica and Durango races of the Mesoamerican genepool (Singh, 1995; Beebe et al., 2013). As with any crop, because of environmental fluctuations and the challenge of reproducing treatments, breeding for drought in common beans is a difficult process (Terán and Singh, 2002*a*; Blum, 2011). The length of time necessary for selection for drought resistance compounds this difficulty, as little benefit exists in selecting for drought resistance in early generations of common beans. The low heritability of seed yield under drought and the large genotype by environment interactions argues that drought selection gains are too small and unreliable to justify the time and cost (Terán and Singh, 2002*b*).

The genetics of common beans are relatively unexplored, especially when compared to a legume crop like soybean. However, previous research to develop genetic markers and deploy them in quantitative trait loci (QTL) for marker-assisted selection has been successful, especially for traits like disease resistance, which are often linked to a smaller number of major-effect genes (Liu et al., 2009; Navarro et al., 2009; Singh and Schwartz, 2010). The resources of common bean genetics are otherwise scarce, and they are especially so for resources related to drought. An early study found marker-assisted selection ineffective for improving yield under drought in common beans (Schneider et al., 1997*a*; Kelly and Miklas, 1998). However, as the tools

available for computation and molecular genetics advanced and allowed for better genomic coverage, recent QTL studies have found SNP markers with a small but significant association with seed yield under drought stress despite the more quantitative nature of drought tolerance (Asfaw and Blair, 2012; Blair et al., 2012; Mukeshimana et al., 2014). Many more drought-related QTLs are likely to be discovered, but of those that have been discovered, few have been linked with their underlying gene(s) (Mukeshimana et al., 2014). The future is promising: complementing the development of genetic markers in common beans are the increasing genomic resources for the crop. The release of the sequenced common bean genome (Schmutz et al., 2014) now provides opportunities for investigation using comparative genomics, transcriptomics, and further marker development, all of which researchers plan to use in bean improvement (McClean et al., 2011). Still, researchers have not yet exploited these new genomic resources. Nonetheless, a survey of drought-related expressed sequence tags (ESTs) in common beans (Blair et al., 2011) helped to support a later study of the drought-activated transcriptome of two varieties contrasting in their tolerance to this stress (Müller et al., 2014). Furthermore, a proteomic analysis of two common bean varieties served to highlight the systemic response to drought stress and its intervarietal and interenvironmental variation (Zadražnik et al., 2013).

Further research is necessary on the genetics and genomics of common beans, and researchers have not yet fully exploited the published genome. Part of the reason is that common beans are recalcitrant to tissue regeneration, so it is difficult to obtain viable transformants for the study of bean genetics. However, the efficiency of sequencing is now so great that the genomes of multiple lines can be compared with each other, and a careful application of this method can be a powerful probe of genetics. An increase in the understanding of common bean stress

physiology, as this dissertation provides, enables forward genetics approaches to investigating the underlying genetic basis for stress response in beans.

Tepary beans

Tepary beans (*Phaseolus acutifolius* A. Gray) are a close relative of common beans native to the Sonoran Desert located in northwestern Mexico and southwestern United States. The appearance of tepary beans closely resembles that of common beans although the former tends to have smaller leaves, more leaves, and a highly-branched, bushy architecture. Like common beans, the size and coat color of tepary bean seeds show variation based on landrace or variety. In the process of adapting to its sub-tropical desert environment, tepary beans developed a high degree of tolerance to heat and drought stresses. The domestication of tepary beans occurred approximately 5,000 years ago, and the crop served as a staple for a number of native cultures in the region, among them the Hopi Pueblos, Papago, Seri, and Mohave (Nabhan and Felger, 1978). Within the genus *Phaseolus*, species are classified within several distinct genepools, with *P. vulgaris* placed in the primary genepool and *P. acutifolius*, based on its evolutionary and genetic distance, placed in the tertiary genepool (Smartt, 1981). Common beans and tepary beans are partially reproductively compatible with each other, requiring embryo rescue after fertilization to obtain viable hybrids (Beebe, 2012). Despite this difficulty, breeders have created a number of common bean lines into which they introgressed tepary bean genetics (Mejía-Jiménez et al., 1994; Singh and Muñoz, 1999). Indeed, some of these introgressed lines gained prominence recently when researchers at CIAT discovered their great capacity for heat tolerance as measured by pollen viability (CGIAR, 2015).

Plant response strategies to abiotic stress

When studying abiotic stress, it is often helpful to place a plant's response to stress in one of three categories: escape, resistance, or tolerance (Levitt, 1972). An escape strategy involves a plant structuring its life cycle so that its quiescent phase corresponds with a period of stress and its growth and reproduction occur when environmental conditions are favorable. For example, desert ephemerals exemplify an escape strategy by rapidly growing, flowering, and setting seed when a rare precipitation event happens; by the time moisture is once again scarce and drought stress would be high, the ephemerals exist as dormant seeds in the soil. However, an escape strategy can also simply be earlier flowering and seed set in a crop exposed to moderate stress. A resistance strategy involves a mechanism that prevents a stress from penetrating a plant's tissues; a high rate of transpiration that lowers a leaf's temperature through latent heat loss would be an example of a resistance strategy to heat stress. Finally, there is the tolerance strategy, in which the plant mitigates adverse effects from stress that does penetrate the tissues; again using heat stress as an example, a plant may produce more heat shock proteins within its leaves when exposed to high temperatures, so while the tissues of the plant do rise in temperature, the heat shock proteins prevent some of the damage that the rise would cause.

These strategies are not always clearly separate from each other, and in many cases, a mechanism for responding to stress could fall into more than one category. However, this classification of stress responses does help when comparing responses, considering how they would interact with each other, and understanding what responses would best aid survival or yield in a given environment. It should be noted that the term "tolerance" is used in this dissertation to describe an organism's general ability to withstand damage from adverse

conditions and, unless noted otherwise, is not used to describe the specific strategy detailed above. As will be seen below, the majority of stress studies in common beans have been conducted in field settings where yield and maturity characteristics are the main parameters being measured. As a result, these studies have investigated the relation between stress escape mechanisms and agronomic success. Yet, stress resistance and stress tolerance mechanisms remain mostly unexplored in common beans. One of the aims of this research is to more fully investigate the resistance and tolerance strategies of common beans as a compliment to the already well-characterized escape mechanisms.

Gas exchange in leaves

Stomata

The means by which plants take up atmospheric carbon dioxide and transport it to rubisco for assimilation have ramifications for the response of plants to water stress. Models of gas diffusion in a leaf include parameters for carbon dioxide concentration in the atmosphere (C_a), in the intercellular leaf spaces (C_i), and within cells at the site of carboxylation (C_c); the diffusion between atmosphere and leaf interior is controlled by stomatal conductance (g_s), and the diffusion between leaf interior and the site of carboxylation is controlled by mesophyll conductance (g_m) (Flexas et al., 2002). Stomata, the pores on leaf surfaces that flanking guard cells can open or close, not only facilitate the diffusion of carbon dioxide into intercellular leaf spaces but also regulate the transpirational loss of water from the leaf. Exposure to water stress upregulates the production of abscisic acid (ABA), and ABA upregulates the production of nitric oxide, which is necessary to conduct the drought signal to guard cells and induce stomatal

closure (Desikan et al., 2002; Neill et al., 2008). The nitric oxide triggers an efflux of K^+ from the guard cells as well as an increase in the cytosolic pH and concentration of Ca^{2+} (McAinsh et al., 1992; Blatt and Armstrong, 1993; MacRobbie, 1998). Other phytohormones involved in the regulation of the stomatal aperture rely wholly on or have crosstalk with ABA (Hossain et al., 2011; Daszkowska-Golec and Szarejko, 2013). However, the pathway responsible for ABA perception and induction of stomatal closure is not the same pathway responsible for inhibiting the opening of stomata (Yin et al., 2013). The basic mechanisms of stomatal control are thus elucidated at the level of general plant physiology. However, few studies have examined these mechanisms in common beans specifically. Examining the variation that exists for these traits and how well they correlate with agronomic success is essential to understanding the physiology of stress tolerance in common beans.

While the loss of water through stomata can be a problem under water stress, it also performs valuable functions. Transpiration creates a gradient in water potential that helps drive the transport of water and nutrients in the vascular tissue and the uptake of soil water by the roots. Additionally, the latent heat loss provided by transpiration increases the yield of plants under high temperatures (Fischer et al., 1998; Araus et al., 2002). This last mechanism creates an antagonistic relationship between how a plant responds to heat and drought stress: it must balance its need to conserve water under drought stress with its need to avoid high temperatures under heat stress.

Gas exchange and photosynthesis

The review by Flexas et al. (2004) summarizes the large body of evidence for stomatal factors and but not metabolic factors being the greater limitation to photosynthesis in plants

experiencing water stress. Stomata are not merely a limiting factor of photosynthesis, but photosynthesis can regulate stomatal aperture; under conditions of limited photosynthesis, such as low irradiance, guard cells will narrow the stomatal openings in response (Wong et al., 1979). Nor are stomata the only barriers to gas diffusion. Under drought stress, *P. vulgaris* leaves maintained high C_i and an unperturbed photosynthetic apparatus at 30% leaf water deficit, yet rates of assimilation were still negligible (Cornic et al., 1989). This phenomenon gives strong evidence for g_m as a second diffusional constraint to photosynthesis that water stress can transiently increase (Delfine et al., 1999; Centritto et al., 2003). How a plant changes its g_m in response to stress helps to determine its water use efficiency (Hommel et al., 2014), and this effect has implications for the study of drought within and between species. While metabolic factors certainly should not be ignored, the above research highlights the importance of diffusive limitations when studying the effects of stress on plants. The research reported in this dissertation probes further the diffusive resistances in beans in order to better understand their physiology under stress.

Photosynthesis, stress, and the problem of excess energy

Excess energy and reactive oxygen species

Stress reduces a plant's ability to fix carbon, but the plant is often still under high solar radiation at various points during the day, so it must safely dissipate this intercepted energy lest reactive oxygen species (ROS) damage the plant (Allen and Ort, 2001; Foyer and Noctor, 2009). Via morphological mechanisms, paraheliotropism allows a plant to reduce the solar radiation it is intercepting, and this response can effectively mitigate stress, especially in beans (Pastenes et al.,

2004; Lizana et al., 2006). However, paraheliotropism can only reduce incident solar radiation to a certain extent, and plants use other mechanisms to ameliorate high light stress. Among these mechanisms is energy dependent quenching (q_E): under excess excitation energy, the pH decreases in the lumen of the chloroplasts, and this acidification drives the xanthophyll cycle conversion of violaxanthin into zeaxanthin, which is necessary for q_E which quenches the energy of excited chlorophyll and dissipates it as heat (Demmig-Adams and Adams, 1996). If this energy is not dissipated, then it can reduce oxygen to form ROS like singlet oxygen, which can in turn be converted to a less reactive state via pathways involving glutathione and ascorbate (Asada, 2006; Foyer and Noctor, 2011).

Although ROS can be damaging when unregulated, they are an integral part of redox signaling within the plant, so plant breeding or genetic engineering strategies should focus more on ROS regulation than their complete prevention (Foyer and Noctor, 2009). For example, cotton plants engineered to have twenty times the level of glutathione reductase activity than wild type still had no photosynthetic or yield advantage in a field setting (Kornyeyev et al., 2005) despite having an advantage under controlled conditions (Kornyeyev et al., 2001, 2003). Before energy dissipation and ROS scavenging traits can be breed into common beans, the strategies of tolerant varieties must first be understood. This dissertation increases the characterization of disparate responses in bean varieties to excess energy and the resulting damage that it causes.

Photorespiration

Photorespiration is another mechanism that is intimately linked to stress and excess energy, and it is especially relevant to C_3 crops like common bean. Because rubisco has affinities for both carbon dioxide and oxygen, it will often perform an oxygenase reaction on ribulose-1,5-

bisphosphate (RuBP) to produce one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate (Bowes et al., 1971). The 2-phosphoglycolate then goes through a series of reactions in multiple organelles that ultimately convert it to 3-phosphoglycerate at the expense of reducing energy and the release of carbon dioxide and ammonia (Ogren, 1984). Some view photorespiration's use of energy, carbon, and nitrogen as inefficient and propose alterations to the photorespiratory pathway, engineering a rubisco with greater affinity to carbon dioxide, or the insertion of carbon concentration mechanisms as ways to increase productivity in C_3 plants (Long et al., 2006; Moroney et al., 2013; Singh et al., 2014).

The photorespiratory pathway is necessary for survival in photosynthetic organisms, and plants deficient in its operation cannot survive under normal atmospheric conditions (Somerville and Ogren, 1982; Timm et al., 2012). Even C_4 plants with compromised photorespiratory pathways will accumulate 2-phosphoglycolate and eventually die when they are exposed to normal atmospheric conditions although these effects can be prevented in low oxygen environments (Zelitch et al., 2009). Thus, photorespiration cannot be wholly removed from plants although careful modifications are still possible. Different groups have used endogenous genes or genes inserted from the bacterium *Synechocystis* to engineer photorespiratory pathways that release carbon dioxide in the chloroplast or avoid the release of ammonia, and researchers are still evaluating these engineered lines (Kebeish et al., 2007; Peterhansel et al., 2013). Instead of viewing it as a waste, it may be equally valid to view photorespiration as a mechanism of dissipating excess energy under conditions of high light (Kozaki and Takeba, 1996). This dissipation would be especially helpful during hot and dry periods when the stomates are closed and the concentration of carbon dioxide at rubisco is low. From this viewpoint, the plant expends carbon to protect itself from photooxidative damage.

As for carbon concentrating mechanisms, when comparing C_3 and C_4 plants, C_4 plants theoretically will only have a photosynthetic advantage at temperatures above 30° C, depending on the species (Ehleringer and Pearcy, 1983). C_4 crops perform better in warmer temperatures in part because they avoid the rise in photorespiration caused by rubisco's increasing ratio of affinity for oxygen versus CO_2 as temperature increases (Jordan and Ogren, 1984). If not from C_4 plants, a carbon concentrating mechanism from algae or cyanobacteria could be engineered into C_3 plants although different barriers to their functioning in C_3 plants exist (Moroney et al., 2013). So even if carbon concentrating mechanisms would not be as much of a boon to C_3 crops in temperate regions, they could perhaps still increase the productivity of such crops in tropical and subtropical areas. However, C_4 metabolism independently arose at least 45 times across a wide range of higher plants (Sage, 2004), but C_4 plants are not ubiquitous in tropical areas, and this lack of ubiquity supports the viability of alternative metabolic strategies in hot environments.

Metabolites and stress

Compatible solutes

Small compatible solutes, namely sugars, sugar alcohols, free amino acids, and quaternary ammonium compounds, are known to play a significant role in many plants' responses to drought stress. Compatible solutes purportedly improve stress tolerance by lowering tissue water potential, stabilizing the structure of proteins, and scavenging reactive oxygen species (Ingram and Bartels, 1996). However, some researchers debate where compatible solutes exert their greatest effect and point out that some of these putative roles are based more on theory than *in vivo* evidence (Hare et al., 1998). Indeed, based on total concentrations,

inorganic ions play a greater role in adjusting water potential than organic compatible solutes in most plants (Hare and Cress, 1997). Hyperaccumulation of compatible solutes is a physiological response most often seen in desiccation tolerant species (Hoekstra et al., 2001), but the mechanisms of desiccation tolerance are not necessarily the same as those for drought tolerance (Serraj and Sinclair, 2002). Indeed, Serraj and Sinclair (2002) were especially critical of previous studies looking at native and genetically engineered effects of compatible solutes, declaring that the importance of these studies were inflated because the experimenters failed to consider the results within the context of agricultural systems, which would never be productive at the low moisture levels used. Although not so bluntly critical, other authors have presented a tempered view of the efficacy of compatible solutes by considering each compound's place within its biochemical pathway and how that placement would affect the wider metabolism of the plant (Hare et al., 1998; Zhang et al., 1999). As will be discussed further below, the research on compatible solutes in common beans is limited, and further research is needed to fully determine the role these may play in stress responses in this species.

Glycinebetaine is an amino-acid derived compatible solute that accumulates in large quantities in certain species (Chen and Murata, 2008) and helps to stabilize protein structure under abiotic stress (Murata et al., 1992). Application of glycinebetaine to common beans does confer greater tolerance to drought than control treatments (Xing and Rajashekar, 1999), but beans produce very little glycinebetaine themselves, especially when compared with a number of stress tolerant species (Storey et al., 1977) or even when compared to Arabidopsis (*Arabidopsis thaliana)* (Xing and Rajashekar, 2001). The amino acid proline also acts as a compatible solute in certain species (Verbruggen and Hermans, 2008), but common beans only have a modest increase in proline concentration under drought stress, and in one study comparing two varieties

differing in stress tolerance, the drought susceptible variety accumulated more proline under stress (Rosales et al., 2012). Few other studies address the role of free proline in drought response in common beans, so one of the aims of this dissertation is to extend the study of proline to a greater number of varieties and conditions to better ascertain proline's effect.

Sugars

Sugar and carbohydrate metabolism also tend to have predictable shifts under drought tolerance. Like with proline and glycinebetaine, the concentration of soluble sugars increases with the incidence of drought stress in a number of species (Souza et al., 2004; Rizhsky et al., 2004; Xiao et al., 2009). Conversely, levels of starch decrease in plants exposed to drought because of a reduction in synthesis caused by the inhibition of photosynthesis (Vassey and Sharkey, 1989; Souza et al., 2004; Muller et al., 2011). Sugars not only reflect the metabolic state of a stressed plant but also act as signaling molecules that control a number of stress-related transcriptional pathways (Gupta and Kaur, 2005; Rolland et al., 2006). For example, the KIN10 and KIN11 transcription factors transfer signals between stress perception and sugar metabolism and regulate starch production under stress (Baena-Gonzalez et al., 2007).

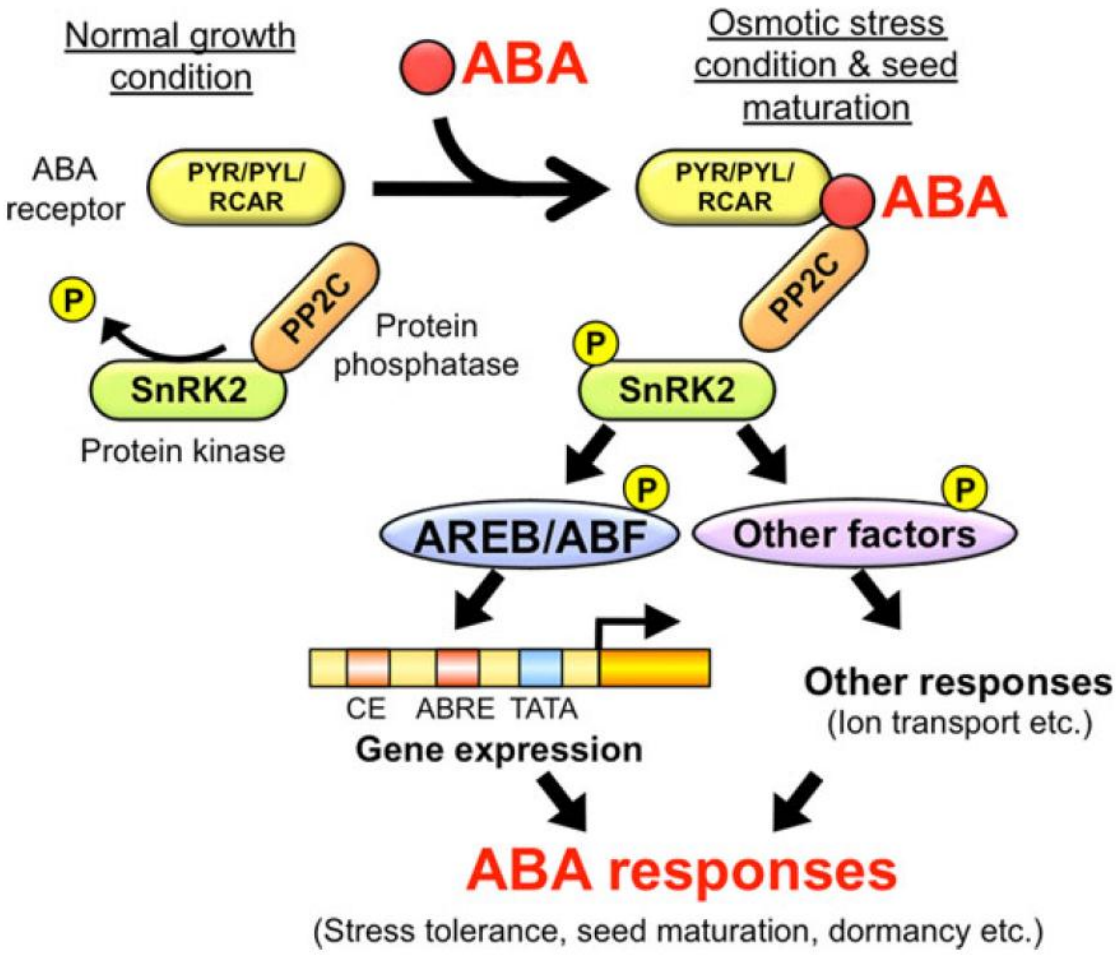
Drought stress initially causes growth rates and carbon metabolism to fall out of sync with each other (Muller et al., 2011). Because photosynthesis will be affected more by diffusive limitations than metabolic ones under drought stress (Flexas et al., 2004), the rate of tissue growth, which depends on cell turgor (Frensch and Hsiao, 1994; Geitmann and Ortega, 2009), may decrease before the rates of photosynthesis do, and this decrease would result in an accumulation in the cell of carbon compounds that are no longer being utilized in the synthesis of structural components. Only one other study (Ramalho et al., 2014) has looked at the

accumulation of sugars in common beans under stress; the work of this dissertation extends the study and examines the concentration of sugars and organic acids that were not included in previous studies.

Abscisic acid

Abscisic acid (ABA) is one of the most important stress signaling hormones in plants. ABA is mainly produced through an offshoot of the carotenoid pathway in plant chloroplasts when nine-cis epoxycarotenoid dioxygenase cleaves violaxanthin to form xanthoxin, the first committed precursor in ABA biosynthesis (Schwartz et al., 1997; Qin and Zeevaart, 1999). The recently discovered interactions between ABA, 2C protein phosphatases (PP2Cs), pyrabactin resistance and pyrabactin resistance-like genes (PYR/PYLs), and a family of SNF1 related kinases (SnRK2s) allowed for the elucidation of the ABA perception pathway (Ma et al., 2009; Park et al., 2009). Normally, PP2C binds to SnRK2, preventing the latter's activity. ABA facilitates the binding of PYR/PYL to PP2C, and this binding releases SnRK2 and allows it to phosphorylate transcription factors to induce their activity (Figure 1). While ABA, SnRK2, and PP2C are all ancient components found in many kingdoms of life, the ABA receptor PYR/PYL arose only in higher plants after they had colonized land (Hauser et al., 2011). After formation, ABA can be reversibly inactivated through conjugation with glucose, or it can be permanently degraded by ABA 8' hydroxylase to form phaseic acid and, further downstream, diphaseic acid (Cutler and Krochko, 1999; Yang and Zeevaart, 2006).

Figure 1 - Abscisic acid perception pathway
Taken from Nakashima and Yamaguchi-Shinozaki (2013), this figure illustrates the major actors of the ABA perception pathway. ABA binds to PYR/PYL, which facilitates the latter's binding to PP2C. This binding releases SnRK2, which then induces the activity of certain transcription factors via phosphorylation.



Historically, some debate existed over whether plants produce ABA primarily in the roots or the shoots in response to stress (Sreenivasulu et al., 2012). A drought signal affecting growth and stomatal response when a part of the root system was placed in dry soil was used as evidence for ABA originating in the root (Hartung et al., 2002; Wilkinson and Davies, 2002). However, the studies using grafting between wildtype and ABA deficient varieties showed that ABA-producing shoot tissue is both necessary and sufficient for ABA accumulation (Holbrook et al., 2002; Christmann et al., 2007). Nonetheless, ABA is transported to the roots and can act as a signaling molecule there as well, so the study of root ABA is justified from a stress physiology perspective. As little research exists on ABA in common beans in general, let alone on tissue specific concentrations of ABA, this dissertation examines these subjects in more detail within the crop of common beans.

Under stress, the flux of material through the ABA synthesis and degradation pathways significantly increases, with a large increase in the breakdown products of phaseic acid and dihydrophaseic acid (Seiler et al., 2011). Some studies have shown that in comparing stress tolerant and stress susceptible varieties within a species, it is the tolerant variety that produces less ABA and ABA breakdown products when exposed to stress (Asch et al., 1995; Seiler et al., 2014). A likely hypothesis is that stress tolerant varieties are able to regulate their response to stress and produce a moderated ABA signal while stress susceptible varieties produce a large, unregulated response via ABA and other signaling molecules that derange the plant's metabolism (Sreenivasulu et al., 2012). The accumulation of ABA in reproductive tissues could be of greater relevance than leaf accumulation when looking at plants from an agricultural perspective. Under drought stress, most genes in the ABA pathway are upregulated in floral tissue except for those related to the catabolism of ABA (Kakumanu et al., 2012). Additionally, the ABA content of

seed heads better correlates with reproductive success than leaf ABA content, and mutant lines deficient in ABA catabolism show reduced yield (Ji et al., 2011). Again, the majority of the research on the agronomic implications of ABA have been done in major cereal crops. Orphan crops like common bean have received little attention in this area. Considering the position of ABA as a master regulator of stress perception and response, the study of ABA in common beans is necessary to a better understanding of its stress physiology. The research in this dissertation was designed to establish a basic understanding of ABA in common beans and how it reacts to drought stress that future studies can use.

Studies of common beans and abiotic stress

Different factors of drought

When breeding common beans for drought tolerance, whether the beans will be in areas with terminal end of season drought or intermittent drought throughout the season influences which traits will be important for their agricultural success (Muñoz-Perea et al., 2006). Higher plasticity of reproductive phase timing provides common beans an advantage under conditions of terminal drought stress (Acosta-Gallegos and White, 1995), but this same plasticity could reduce yield in an intermittent drought setting. Perhaps just as important as the type of drought is the physiological stage of the bean plant when drought occurs. Drought that occurs during the reproductive phase has a much more negative impact on yield than drought that occurs during vegetative or grain-filling stages (Nielsen and Nelson, 1998). Carbon isotope discrimination assays on a collection of diverse common bean lines revealed significant differences in water use efficiency based on the region the lines bred for but not on ancestral genepool (Ehleringer et al.,

1991). Nonetheless, materials adapted to one type of drought may still be of value for breeding in the other type of drought environment (Frahm et al., 2004).

Metrics related to yield

A field study of common beans under nonstress and drought conditions showed no clear correlations between yield and water use efficiency, total biomass, or harvest index under either treatment (Muñoz-Perea et al., 2007); rather, high-yielding varieties were able to compensate with a high score in one metric for a deficiency in another. This observation makes sense given the mathematical interrelatedness of the terms:

$$Y = T \times WUE \times HI \quad (1)$$

where Y is yield, T is transpiration, WUE is water use efficiency in terms of weight aboveground biomass per amount of water transpired, and HI is harvest index (Passioura, 1996). Blum (2009) argues that water use efficiency is an unhelpful target for plant breeders and that the concept of effective use of water better represents the agronomic yield of crops in water-limiting conditions, but as long as its mathematical relationship to yield is known, water use efficiency can still serve as a helpful summary number.

In a study examining the yield of common beans under terminal drought and intermittent drought conditions, harvest index decreased slightly in response to drought while yield strongly correlated with aboveground biomass and strongly negatively correlated with days until flowering for both drought types (Rosales-Serna et al., 2004); these results support overall plant vigor under drought and escape from drought through earliness as the greatest contributors to

yield under stress. Early maturity correlating positively with yield under drought stress has been observed in other studies (Singh, 1995; Terán and Singh, 2002*a*). When breeders select for drought tolerance, they want to also make sure that they are not inadvertently selecting against yield under well-watered conditions. For two quantities, their geometric mean is the square root of their product. The geometric mean of a cultivar's yield under drought stress and yield under well-watered conditions in the same environment has proven a simple way to help identify which lines show superior performance under both conditions (Schneider et al., 1997*b*; Terán and Singh, 2002*a*; Frahm et al., 2004). The geometric mean can be a helpful metric in heat stress experiments as well (Porch, 2006).

Physiological factors in stress response of beans

Although some varieties yield well in all conditions (Terán and Singh, 2002*a*; Rao et al., 2013), field yields under well-watered conditions were poorly correlated with yields under drought conditions as varieties yielding well under drought stress are surpassed by other varieties under well-watered condtions (Terán and Singh, 2002*b*; Lizana et al., 2006). In one study (Lizana et al., 2006), the tolerant genotype maintained its growth rate and abscised fewer of its reproductive organs under drought stress while the susceptible genotype had large rates of floral and pod abscission. Additionally, soil and leaf water status more strongly controlled stomatal aperture than light levels in the tolerant genotype while the reverse was true for the susceptible genotype (Lizana et al., 2006). In another study using the same two genotypes exposed to different light intensities, the tolerant genotype was able to utilize higher light intensities for photosynthesis, and this utilization correlated more with plasticity of stomatal development than with accumulation of carotenoids (Wentworth et al., 2006).

In a proteomic study of common beans under drought stress, when compared to the susceptible genotype, the tolerant genotype had more abundant changes in proteins related to photosynthesis and fewer in proteins related to stress response and ROS scavenging (Zadražnik et al., 2013). Interestingly, the strongest individual protein group contrast was that the tolerant genotype down regulated its abundance of oxygen evolving enhancer proteins (OEEs) while the susceptible genotype upregulated all of its OEEs under drought stress. Hypothetically, the difference in abundance of OEEs could represent the broader stress response strategies of these genotypes: while the susceptible variety enhances its photosynthetic capacity in response to stress and dangerously uncouples its available reducing energy from its carbon supply, the tolerant variety reduces its photosynthetic capacity and thus dissipates the incident energy in other ways. In comparisons of three bean genotypes under drought stress, the genotype that showed the greatest increase in lipid peroxidation and membrane disruption also had higher amounts of ROS and lower activity for ROS-scavenging enzymes (Zlatev et al., 2006). A similar experiment using the same genotypes studied chlorophyll fluorescence, gas exchange, metabolite, and water potential parameters (Ramalho et al., 2014). The significant difference among genotypes was that the high ROS line from Zlatev et al.'s (2014) experiment also had lower rates of photosynthesis, stomatal conductance, and photosystem II efficiency under drought stress, but it also had higher levels of carotenoids (Ramalho et al., 2014). Water deficits also reduce common beans' capacity to fix nitrogen although resistance to this reduction exists within the germplasm (Devi et al., 2013).

Different cultivars have different rates of pollen derangement under heat stress with commensurate rates of pollen viability and seed yield per plant (Porch and Jahn, 2001). Although increases in temperature only moderately affected rates of photosynthesis and conductance, it did

severely reduce pollen viability and seed set, and increasing the ambient carbon dioxide concentration could not compensate for stress induced by heat (Prasad et al., 2002). These findings suggest that the warmer global temperatures of the future will restrict the growth of common beans in production zones as the higher carbon dioxide concentration will do little to improve pollen viability. The recent release by the International Center for Tropical Agriculture (CIAT) of a number of heat tolerant common bean lines (CGIAR, 2015) may allow farmers to maintain production in the areas where they grow common beans over the coming decades.

The relative contributions of shoot tissue and root tissue to drought response is an issue of much discussion. In a grafting experiment involving several different lines of common beans, White and Castillo (1992) found that root identity had the largest impact on small plot yield under water-limiting conditions. Shoot identity affected days to maturity, but the authors considered the shoot's effect on seed yield to be insignificant. Sponchiado et al. (1989) also concluded that common bean's yield under drought stress is primarily associated with rooting depth, but this association was not true across all sites that they tested, and the drought susceptible varieties accumulated near identical weights of above ground biomass as the tolerant varieties.

Other factors

While a crop in a field setting is sometimes affected by just one stress, in many cases, a crop will undergo multiple coincident stresses. These stresses can exacerbate each other; for example, high temperatures will damage a drought stressed plant more because it lacks the water to cool itself through evapotranspiration. Most studies focus on a single stress because it is easier to draw conclusions about the effects of that stress when it is the only variable being

manipulated. However, a plant's biochemical and transcriptional response to two coincident stresses can be substantially different from its responses to those stresses individually (Rizhsky et al., 2004; Rasmussen et al., 2013).

One inadvertent form of stress in greenhouse and growth chamber experiments comes from an inadequate consideration of root conditions (Poorter et al., 2012*b*). Small pot sizes limit root growth and can negatively impact photosynthesis while using improper soil media and watering regimes can place the roots in a near constant state of hypoxia (Passioura, 2006; Poorter et al., 2012*a*). In designing experiments, Poorter (2012a) recommends not exceeding 2 g plant biomass per L of soil volume.

Comparisons of common bean and tepary bean

Common bean and tepary bean have significantly diverged for some of their responses to stress to adapt to their respective native environments. Nonetheless, the two species are closely related, and their shared genetics is evidenced by their similarities in morphology and metabolism. Common bean and tepary bean can both follow a drought escape strategy of early maturity during a late season terminal drought although this escape strategy is not ubiquitous in former as it is in latter (Nabhan and Felger, 1978; White and Singh, 1991). Exposure to high temperatures for prolonged periods completely prevents seed set in many common bean lines, but many tepary beans are able to yield seed, albeit a reduced yield, at the same extreme temperatures (Rainey and Griffiths, 2005*b*). In a field experiment tepary bean lines yielded more under water limited conditions and fair to excellent under irrigated conditions in comparison with all the common bean lines tested (Rao et al., 2013). However, the more drought tolerant

common beans were closer in performance to tepary beans than to other common bean lines under drought stress (Rao et al., 2013). Interspecific inbred backcross lines yielded poorly under both conditions, and this poor performance raises concerns about the ease with which drought tolerance from tepary bean can be bred into common bean. The total aboveground biomass of the tepary lines was not substantially different from biomass of the common bean lines, so the high yield of the tepary bean lines results in part from their superior harvest index under drought conditions (Rao et al., 2013).

When comparing the two species, tepary bean's root systems grows to greater depth, and tepary bean reduces its stomatal conductance more severely at higher leaf water potentials (Markhart, 1985). Conversely, tepary bean lost its root length advantage when exposed to soil aluminum stress, and even under combined aluminum and drought stress, tepary bean had lower root lengths than common bean (Butare et al., 2011). It is likely that tepary bean is more sensitive to metal toxicity in general. In drought experiments featuring grafts between common bean and tepary bean, leaf water potential associated most closely with root identity, and plants with a tepary rootstock tended to have higher leaf water potentials (Sanders and Markhart, 1992). These results support the hypothesis that tepary bean has higher root hydraulic conductivity than common bean. However, when increased osmoticum in the watering solution was used as the source of water stress and when cuticular barriers to diffusion were removed, both species had similar leaf water potentials and photosynthetic responses (Castonguay and Markhart, 1991). A similar experiment measuring photosynthesis in intact leaves revealed that tepary bean had a higher carboxylation efficiency and a higher water use efficiency than common bean under water stress (Castonguay and Markhart, 1992). While both common bean and tepary bean exhibit paraheliotropic movement of their leaves, tepary bean increases its leaf angle to a greater degree

under conditions of high light and temperature (Bielenberg et al., 2003). Studies on ABA levels within the genus *Phaseolus* have also been carried out. During extended exposure to salt stress, common beans have increasing levels of leaf ABA with increasing levels of salinity, but tepary beans show no increase in leaf ABA content, whatever the salt level (Yurekli et al., 2004). Tepary bean accumulates more ABA in its leaves under heat stress than either a heat tolerant common bean line or a heat susceptible one (Udomprasert et al., 1995).

Overall, tepary bean uses a mix of increased resistance and tolerance strategies that allow it to perform better under stress than common beans; tepary bean's increased rooting depth and reduced stomatal conductance during stress serve to place more water in its tissues, and its increased carboxylation efficiency allows it to better tolerate a decreased CO_2 supply caused by closed stomates.

The research of this dissertation

As examined in this review, common beans are an important crop for many regions, but drought stress often limits yields and heat stress limits areas of production. Building off previous research in beans and other species, this dissertation examines the physiological response to drought or heat stress of a small number of bean genotypes that differ in their tolerance to abiotic stress. The effect of drought on concentrations of proline, carbohydrates, and abscisic acid is examined in Chapter 2. The relation of drought tolerance to photosynthetic and leaf characteristics is examined in Chapter 3. Finally, Chapter 4 examines the effects of high temperatures on photosynthesis and indicators of stress on a larger group of bean germplasm.

Chapter 2: Drought stress and metabolites

Introduction

Common beans (*Phaseolus vulgaris*) are important food crops in the United States, Central America, South America, and East Africa (Akibode and Maredia, 2011) and are the most widely grown legume for direct human consumption (FAOSTAT, 2014). Beans are a nutritious foodstuff, and in addition to being high in quality protein, they provide the nutrients iron and zinc in appreciable quantities as well. Deficiencies in these two nutrients are common throughout the world (Fletcher et al., 2004), so greater access to and consumption of beans are relevant to the public health of both developed and developing countries. Drought stress is among the greatest limiting factors to the production of common beans (Beebe et al., 2013), so breeding drought tolerant cultivars would help create a more stable supply of beans.

A greater understanding of bean's physiological response to drought stress helps inform plant breeders of those traits to focus on during the selection process. A number of metabolites are known to be involved in the abiotic stress response in plants. The amino acid proline was shown to accumulate in Arabidopsis in response to cold and drought stress (Wanner and Junttila, 1999; Urano et al., 2009), but the extent of variation among tolerant and susceptible genotypes in the model organisms for proline accumulation remains understudied (Verslues and Juenger, 2011). Proline also accumulated in crop plants such as wheat and rice experiencing osmotic stress (Garcia et al., 1997; Nayyar, 2003), and exogenous proline alleviated salt stress imposed on suspension-cultured tobacco cells (Hoque et al., 2007).

Sugar metabolism also changes in response to drought stress. As drought stress increases and photosynthesis decreases, a smaller supply of photosynthate is available to developing reproductive tissues, which can lead to their abortion (Pinheiro and Chaves, 2011); supplying

sucrose to drought stressed maize ears rescued their seed yield (Zinselmeier et al., 1995). However, at moderate levels of stress, the supply of soluble carbohydrates often increases in leaves because of the cessation of growth and export (Chaves and Oliveira, 2004). In maize, drought stress affected the transcriptional and metabolic profiles of many sugars (Witt et al., 2012; Kakumanu et al., 2012). The accumulation of sugars under drought stress is thought to result in osmotic adjustment of plant tissues (Levitt, 1972; Morgan, 1984), but the degree to which sugars contribute to osmotic adjustment is questioned, especially when compared to contribution by inorganic solutes (Hare and Cress, 1997; Hare et al., 1998; Serraj and Sinclair, 2002; Gagneul et al., 2007). Perhaps just as important to drought stress, the concentrations of certain soluble carbohydrates act as signals of stress and metabolic status (Carrari et al., 2004; Koch, 2004; Smith and Stitt, 2007; Usadel et al., 2008; Pinheiro and Chaves, 2011). Although sugar responses in common beans are not well studied, Ramalho et al. (2014) measured an increase in soluble sugars in leaf tissues when bean plants were exposed to drought stress.

Abscisic acid (ABA) is the major plant hormone for signaling abiotic stresses, especially drought stress (Wilkinson and Davies, 2002). Upon exposure to drought stress, a plant's concentration of ABA increases, which in turn causes the stomata to close by triggering an efflux of ions from the guard cells (Mori and Murata, 2011). While the closure of stomata conserves water, it also limits photosynthesis by decreasing the influx of carbon dioxide to sites of carbon fixation (Chaves, 1991). ABA is thus critical to determining the balance between productivity and water loss. However, drought tolerance is not simply a matter of accumulating ABA as no relation exists between ABA concentration under drought stress and a plant's degree of drought tolerance (Chaves and Oliveira, 2004). Rather, a plant's survival and productivity under drought stress depends on the maintenance of ABA homeostasis (Sreenivasulu et al., 2007). While ABA

has been well studied in Arabidopsis and cereal crops (Tian et al., 2004; Harb et al., 2010; Kanno et al., 2010; Ji et al., 2011; Kakumanu et al., 2012; Yin et al., 2013; Seiler et al., 2014; Dalal and Inupakutika, 2014), a dearth of information on ABA in beans exists, despite the crucial step in ABA biosynthesis first being discovered in common bean (Qin and Zeevaart, 1999).

The current study used a diverse group of bean genotypes contrasting in their degree of drought tolerance, including the drought tolerant related species tepary bean (*Phaseolus acutifolius*), to investigate the role of several metabolites in drought response in beans. The concentration of the amino acid proline, the concentration of several carbohydrates, and the concentration of, and sensitivity to, ABA were all measured in a range of bean genotypes.

Materials and methods

Preliminary metabolite screen

To determine which compounds were associated with drought response in beans, a preliminary metabolite screen was performed. Plants were grown in a growth chamber at a temperature of 25°C during the light period and 20° C during the dark period, and the chamber ran according to a 14 h light and 10 h dark cycle. Light intensity was 400 µmol photons m^{-2} s^{-1} PAR during the day cycle. Plants were planted in 10 L black plastic pots filled with Suremix perlite potting media (Michigan Grower Products, Galesburg, Michigan). Common bean genotypes Jaguar, Zorro, and SER-16 were used in the experiment as well as the improved tepary bean line TB1 provided by Dr. Timothy Porch from USDA-ARS in Mayaguez, Puerto Rico (Porch et al., 2013). Jaguar and Zorro are two elite black bean cultivars bred in Michigan by Michigan State University; Jaguar is an older cultivar that Zorro completely replaced

commercially after the latter's release because of its higher and more stable yield (Kelly et al., 2009*a*). SER-16 is a small red bean line bred in Colombia by CIAT as part of their development of abiotic stress tolerant lines, and it has shown tolerance to stress in field settings (Beebe et al., 2008; Rao et al., 2013). In terms of drought tolerance, Jaguar is a susceptible genotype, Zorro is moderately tolerant, SER-16 is tolerant, and tepary is very tolerant.

Several seeds of each of the four varieties were sown in the same pot, and after germination, the seedlings were thinned so that each pot contained four plants representing one plant of each variety. Two water treatments were imposed on the plants: well-watered and drought stress, and both treatments had six replicates each. The pots were arranged in a randomized complete block design with pots as the blocking factor. After sowing, all plants were regularly watered with half-strength Hoagland solution mixed with a soluble fertilizer to bring the final nitrogen concentration of the solution to 15 mM. All plants developed mature trifoliate leaves within four days of each other, and after the last plant had matured, water was withheld from the drought stress treatment while the well-watered treatment continued to be regularly supplied with deionized water. After five days of withholding water, the plants in the drought stress treatment were wilting, so one leaflet was harvested from all plants by using a razor to cut the leaflet from the petioule, and the harvested leaflets were flash-frozen in liquid nitrogen, stored at -80° C, and finally lyophilized.

After lyophilization, the samples were finely ground using a Wiley Mill (Thomas Scientific, Swedesboro, New Jersey, USA) and weighed, and approximately 100 mg of each sample was then extracted with 2 mL of 80% ethanol for 30 minutes at 65°C. Then, the samples were centrifuged, and the supernatant decanted. The remaining pellets were extracted twice more as above, and the supernatants were pooled and 3 mL of water added to each one. Then, 3 mL of

chloroform was added to the supernatant, they were vortexed together, and the phases were allowed to separate overnight in a 4 °C refrigerator. The next day, the aqueous upper portion was pipetted into a separate tube and completely dried down on a Speedvac with minimal heat. Dry samples were resuspended in 0.5 mL of pyridine containing 30 mg/mL hydroxylamine hydrochloride and 1 mg/mL xylitol (as an internal standard) and placed on a heating block set to 75° C for one hour. After the samples cooled, 1 mL of hexamethyldisilazane was added to each sample followed by 0.1 mL of trifluoracetic acid. The samples were vortexed and allowed to sit for one hour. Twelve leaf extracts, taken evenly from the genotypes Jaguar and tepary and the drought stressed and control treatments, were then taken to Michigan State University's Mass Spectrometry and Metabolomics Core, and the compounds contained in them were identified using gas chromatography-mass spectrometry. Compounds accumulating in large quantities in any of the samples were marked for further study.

Proline content in stressed leaves

Bean plants were grown in a growth chamber on a 14 hour light and 10 hour dark cycle, with 300 µmol photons m^{-2} s^{-1} of PAR supplied by a combination of fluorescent and incandescent lamps during the light period. Temperature was maintained at 21° C. Plants were grown in 1 L black plastic pots filled with Suremix Pearlite potting media (Michigan Grower Products, Galesburg, Michigan). As leaf samples were taken from young plants, these pots had sufficient capacity for the plants' small sizes.

Four different common bean genotypes were used in this experiment: Jaguar, Zorro, SER-16, and RAB-651. With the first three genotypes described above in 'Preliminary metabolite screen', RAB-651 is a red bean line bred in Colombia by CIAT as part of their

development of abiotic stress tolerant lines, and it has also shown tolerance to stress in field settings (Beebe et al., 2008; Rao et al., 2013). Each variety was sown into two different water treatments: well-watered and drought stressed. Each variety by treatment combination had six pots assigned to it, with one plant per pot, and the plants were grown in a randomized complete block design.

All pots were irrigated with half-strength Hoagland solution and kept well-watered until the first trifoliate leaf was fully mature, and then were subjected to differential water treatments. Three days elapsed between the first plants to have mature trifoliates and the last plants to have mature trifoliates, and the watering treatments did not begin until these last plants were fully mature. The well-watered treatment continued to be watered with deionized water and maintained between 60-100% of pot water-holding capacity (pot capacity) as determined by weighing the pots. Water was withheld from the drought stressed treatment for three days, at which point, their pot capacity was in the range of 20-30%. Then, one leaflet from the first trifoliate leaf of each plant was cut with a razor at the connection of petioule to leaf blade, immediately frozen in liquid nitrogen, and stored at -80° C.

All leaf samples were lyophilized and proline content was determined according to (Bates et al., 1973) with the following modifications. Briefly, each leaf sample was ground using a Wiley Mill, weighed, and then extracted with 0.5 mL of 3% sulfosalicylic acid four times, and the homogenate was pooled and centrifuged. Acid ninhydrin was made by combining 1.25 g of ninhydrin with 30 mL of glacial acetic acid and 20 mL of 6 M phosphoric acid until dissolved. The homogenate of each sample was then reacted with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin for 1 hour at 100° C. The samples were then cooled in an ice bath, extracted with 4 mL of toluene, and the absorbance of the proline containing toluene was measured at 520 nm

using a spectrophotometer. Pure toluene was used as a blank, and the proline concentration of each sample was determined from a standard curve.

All statistical analysis was performed using the program SAS (SAS Institute, Cary, North Carolina). The data was analyzed as a two-factor randomized complete block design using the code PROC MIXED. For factors that were significant, the individual treatment means were compared using Least Significant Difference. An alpha value of 0.05 was set as the threshold for which results would be considered statistically significant.

Soluble carbohydrates in bean leaves

Plants were grown in a growth chamber using the same environmental conditions as described above in 'Preliminary metabolite screen'. Several seeds of each of the genotypes Jaguar, Zorro, SER-16, and tepary were sown in the same pot, and after germination, the seedlings were thinned so that each pot contained four plants representing one plant of each variety. Two water treatments were imposed on the plants: well-watered and drought stress, and both treatments had six replicates each. The pots were arranged in a randomized complete block design with pots as the blocking factor. After sowing, all plants were regularly watered with half-strength Hoagland solution mixed with a soluble fertilizer to bring the final nitrogen concentration of the solution to 15 mM. All plants developed mature trifoliate leaves within three days of each other, and after the last plant had matured, water was withheld from the drought stress treatment while the well-watered treatment continued to be regularly supplied with deionized water. After five days of withholding water, the plants in the drought stress treatment were wilting, so one leaflet was harvested from all plants by using a razor to cut the leaflet from

the petioule, and the harvested leaflets were flash-frozen in liquid nitrogen, stored at -80° C, and finally lyophilized.

After lyophilization, the samples were finely ground using a Wiley Mill (Thomas Scientific, Swedesboro, New Jersey, USA) and weighed, and approximately 100 mg of each sample was then extracted with 2 mL of 80% ethanol for 30 minutes at 65°C. Then, the samples were centrifuged, and the supernatant decanted. The remaining pellets were extracted twice more as above, and the supernatants were pooled and 3 mL of water added to each one. Then, 3 mL of chloroform was added to the supernatant, they were vortexed together, and the phases were allowed to separate overnight in a 4 °C refrigerator. The next day, the aqueous upper portion was pipetted into a separate tube and completely dried down on a Speedvac with minimal heat. Dry samples were resuspended in 0.5 mL of pyridine containing 30 mg/mL hydroxylamine hydrochloride and 1 mg/mL xylitol (as an internal standard) and placed on a heating block set to 75° C for one hour. After the samples cooled, 1 mL of hexamethyldisilazane was added to each sample followed by 0.1 mL of trifluoracetic acid. The samples were vortexed and allowed to sit for one hour. The concentration of compounds identified previously in the metabolite screen was analyzed for each sample using gas chromatography.

Starch determination in bean leaves

Starch levels were determined from leaf samples taken from an identical replication of the 'Soluble carbohydrates in bean leaves' experiment. The concentration of soluble sugars in leaf samples from this experiment was measured as described in 'Soluble carbohydrates in bean leaves', and both experiments had similar concentrations of soluble carbohydrates. The procedure to measure starch was carried out according to the protocol found in Ebell (1969). Briefly, the

pellets remaining from extraction of soluble carbohydrates with ethanol were dried on a Speedvac. 2 mL of acetate buffer was added to each sample and reacted for an hour at 100° C. After cooling, 0.1 mL of an amyloglucosidase solution was added to each sample, and the samples were incubated for 16 hours at 55° C. An aliquot of 80 µL from each sample was taken and deionized water added to it to make a final volume of 1 mL. Each sample was split into three replications of 0.25 mL each, and 2 mL of a glucose oxidase, peroxidase, and O-dianisidine dihydrochloride color reagent was added to each replication (Keller and Loescher, 1989). After sitting for 40 minutes, the reaction was stopped by adding 2 mL of 6 M sulfuric acid. Absorbance of the samples was then read at 540 nm, and concentrations were determined from a standard curve of glucose ranging from 0 to 80 µg/mL, and the technical replicates' readings were averaged to represent the sample.

Application of abscisic acid

The three common bean genotypes, Jaguar, SER-16, and Zorro, and TB1 tepary were grown in a growth chamber set to 25° C, 400 µmol photons m^{-2} s^{-1} PAR light intensity during the day, and 14 hour light / 10 hour dark periods. Seeds were sown into 10 L black plastic pots filled with Suremix pearlite mix potting media (Michigan Grower Products, Galesburg, Michigan, USA). After germination, seedlings were thinned to one per pot. Six replicates of each variety were planted. Pots were fertilized with 5 g of Osmocote 15-9-12 slow-release fertilizer (Bloomington Brands, LLC, Bloomington, Indiana, USA) and supplemented once a week by watering with half-strength Hoagland solution until the pots were saturated and dripping.

When the first trifoliate leaf was fully expanded, the treatments with ABA began. Treatment solutions consisted of a 0.01% (volume/volume) solution of the surfactant Tween 20

mixed with enough of the commercial ABA product ConTego Pro SL (donated by Valent Biosciences, Libertyville, Illinois, USA) to create a solution with a known ABA concentration. On the first day of treatments, in the afternoon, the plants were sprayed to drip with the Tween solution with no ABA. The following day, in the mid-morning, the stomatal conductance of the youngest fully mature leaf was measured using the LI-COR 6400XT portable gas exchange analyzer (LI-COR Biosciences, Lincoln, Nebraska). The conditions inside the LI-COR 6400XT's measuring chamber were 1000 µmol photons m^{-2} s^{-1} PAR, 400 µmol mol^{-1} CO_2, a relative humidity ranging from 52-58%, and block temperature ranging from 26-30 °C. This pattern of spraying in the afternoon and measuring the following mid-morning continued using a 0.1 mM solution of ABA, then a 0.5 mM solution of ABA, and finally a 1 mM solution of ABA. The same group of plants were being sprayed each time, so the plants were exposed to progressively increasing concentrations of ABA. The experiment was carried out over four days to minimize the effects of leaf age on stomatal conductance.

Statistical analysis was done in SAS (SAS Institute, Cary, North Carolina) using the PROC MIXED procedure. The experiment was analyzed as a completely randomized design with ABA as a repeated measures variable.

Determination of endogenous abscisic acid

The bean lines Jaguar, SER-16, tepary, and Zorro were planted and grown in growth chamber conditions similar to those described above in the 'Application of abscisic acid' section but with the following modifications. The bean lines were grown in a common pot setup: each 10 L black plastic pot contained one plant of each of the four bean lines. The well-watered treatment and the drought stressed treatment each had six common pot replications. When the third trifoliate leaf had fully expanded in the common bean lines, watering was stopped for the

drought stressed treatment while continuing for the well-watered treatment. One day after the start of differential watering, in the morning, a 17 mm diameter leaf punch was taken from the latest fully expanded leaf of each plant.. Two days after the start of differential watering, the drought stressed treatment was wilting, and leaf punches were taken from all plants as before. Three days after the start of differential watering, watering of the drought stressed treatment resumed, and leaf punches were taken four and five days after differential watering, with these two days representing the recovery period for the drought stress treatment plants.

To analyze the leaf punches, a solution consisting of 70% methanol, 30% water, 0.1% formic acid (volume / volume), and 0.1 µM concentration of labeled ABA^{d6} (an internal standard containing six deuturated hydrogens) was used for extracting ABA from plant tissue. Each leaf punch taken above was placed directly into 1 mL of extraction solution and left to extract overnight at 4° C. The following morning, the samples were centrifuged for 5 minutes at 5000 g, the supernatant was collected and evaporated to dryness on a Speedvac, and the resulting pellet redissolved in 100 µL of 50% methanol. The redissolved samples were quantitatively analyzed for ABA concentration using a protocol developed by Dr. Dan Jones using tandem ultra high performance liquid chromatography and mass spectrometry with negative ion electrospray at the Mass Spectrometry and Metabolomics Core of the Research Technology Support Facility at Michigan State University.

For determining endogenous levels of ABA in roots and shoots of grafted bean plants, plants were grown and treated as above but with the following differences. The varieties Jaguar and TB1 tepary were grown as they represented the two extremes of susceptibility and tolerance to drought. They were initially sown in two flats of eighteen 10 cm diameter pots, with two seeds per pot. Three days after germination, plants were grafted in four combinations: Jaguar shoot

grafted onto a Jaguar root, Jaguar shoot on tepary root, tepary shoot on Jaguar root, and tepary shoot on tepary root. Plants were grafted used a sharp razor blade to cut the stems midway between the soil and the unifoliate leaves. The shoot's stem end was pared on two sides to form a pointed wedge, and then a vertical cut was made 1 cm deep into the top of the rootstock's stem. The shoot wedge was inserted into the rootstock stem's cut so that the cut sides of both were in full contact with each other, and the entire graft junction was then wrapped in a small piece of Parafilm. A bag was placed over each plant to maintain a humid environment, and the plants were kept well-watered in the growth chamber for two weeks before the bags were removed and the plants transferred to 10 L common pots with one of each graft type in the same pot, six of these common pots total. After a month of normal growth, water was withheld from all plants for six days to impose drought stress, at which point the photosynthesis and stomatal conductance of all plants were near zero. It was assumed that when gas exchange was near zero, the plants were experiencing severe stress, but leaf ABA concentrations actually were closer to a period of moderate stress (Figures 5 and 6). Leaf samples for ABA determination were taken as above. Plants were then uprooted, the roots quickly washed twice in a mild detergent solution (1% w/v solution of Alconox powdered detergent (Alconox Inc., White Plains, New York, USA)) to remove soil, and the crown and tap roots frozen in liquid nitrogen and later lyophilized before determination of ABA as above.

Results

Preliminary metabolite screen

Gas chromatography-mass spectrometry identified a multitude of compounds in the leaf extracts (Supplemental Data 1). Compounds that accumulated in appreciable concentrations, as determined from their percentage of total peak area, were considered for further analysis. The compounds so identified were malic acid, glucose, fructose, inositol, sucrose, and raffinose.

Proline content in stressed leaves

No significant differences were found in leaf proline content between the well-watered control and the drought-stressed treatment, nor were there any significant differences in leaf proline content among the four common bean varieties tested (Figure 2). Proline levels for all variety by treatment combinations were statistically indistinguishable from each other.

Soluble carbohydrates and starch in stressed bean leaves

For most all soluble carbohydrates, drought stress caused a significant increase in their concentration in bean leaves. Malic acid increased roughly four-fold to 4 mg per g of dry tissue in plants exposed to drought stress, but no differences were found among genotypes (Figure 3A).

For fructose (Figure 3B), tepary and Zorro had significantly higher concentrations than Jaguar or SER-16 under drought stress although all genotypes had similarly low levels of fructose under well-watered conditions. Glucose followed a similar pattern (Figure 3C) with indistinguishable and low levels under well-watered conditions and a significant increase under drought stress, but compared to fructose, glucose was generally present in greater concentrations

Figure 2 - Proline content in bean leaves
The amount of proline in leaves of different bean genotypes in exposed to drought stress. Blue bars represent the amount of proline in leaves of the well-watered control, and red bars represent proline levels in the leaves of the drought stress treated plants. Within the well-watered treatment, bars that do not share any lowercase letters are significantly different from each other, and within the drought stressed treatment, bars that do not share any uppercase letters are significantly different from each other (alpha=0.05). Error bars represent standard error.

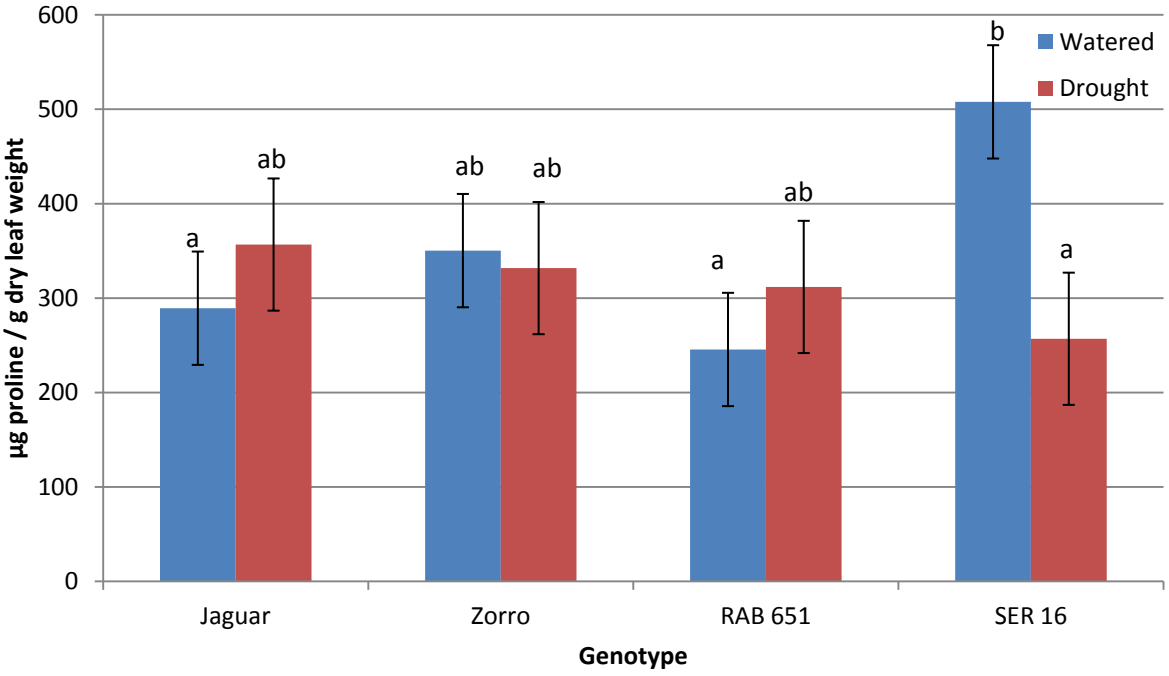


Figure 3 - Soluble organic acids and carbohydrates in bean leaves
The amount of (A) malic acid, (B) fructose, (C) glucose, (D) inositol, (E) sucrose, (F) raffinose, and (G) starch present in the leaves of four different bean lines exposed to drought stress (red bars) or maintained in a well-watered condition (blue bars). Letters located above bars are used to indicate significant differences among lines within a stress treatment; bars that share no letters are significantly different from each other (alpha= 0.05). Error bars represent standard error.

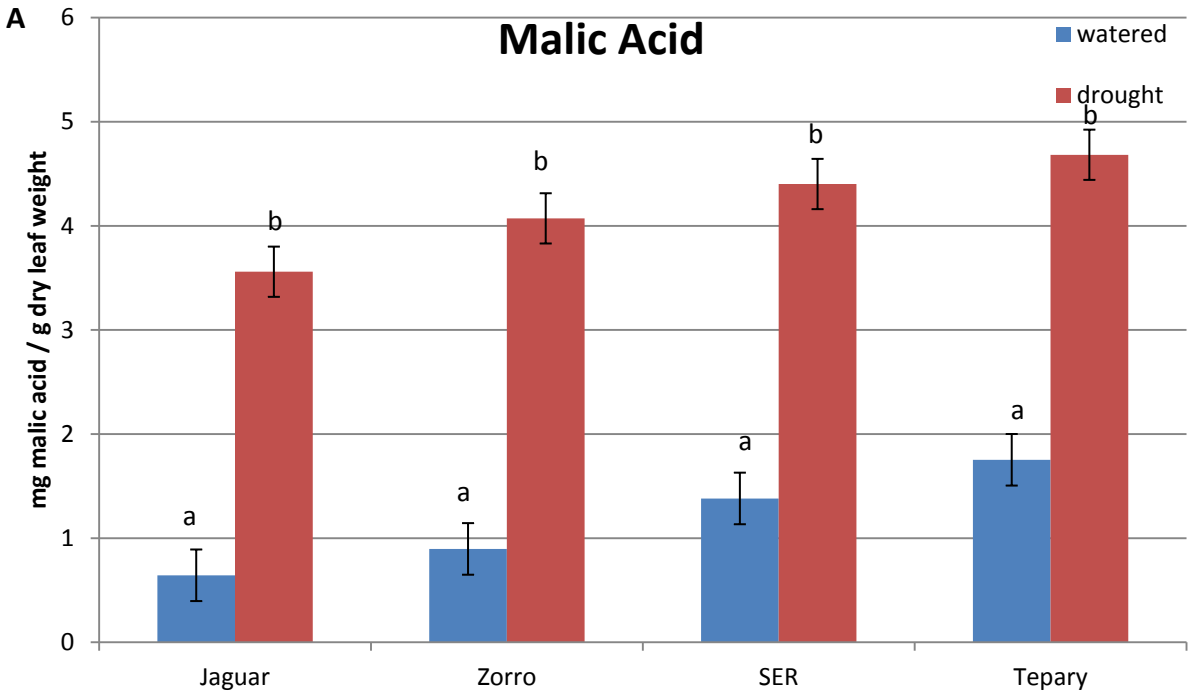
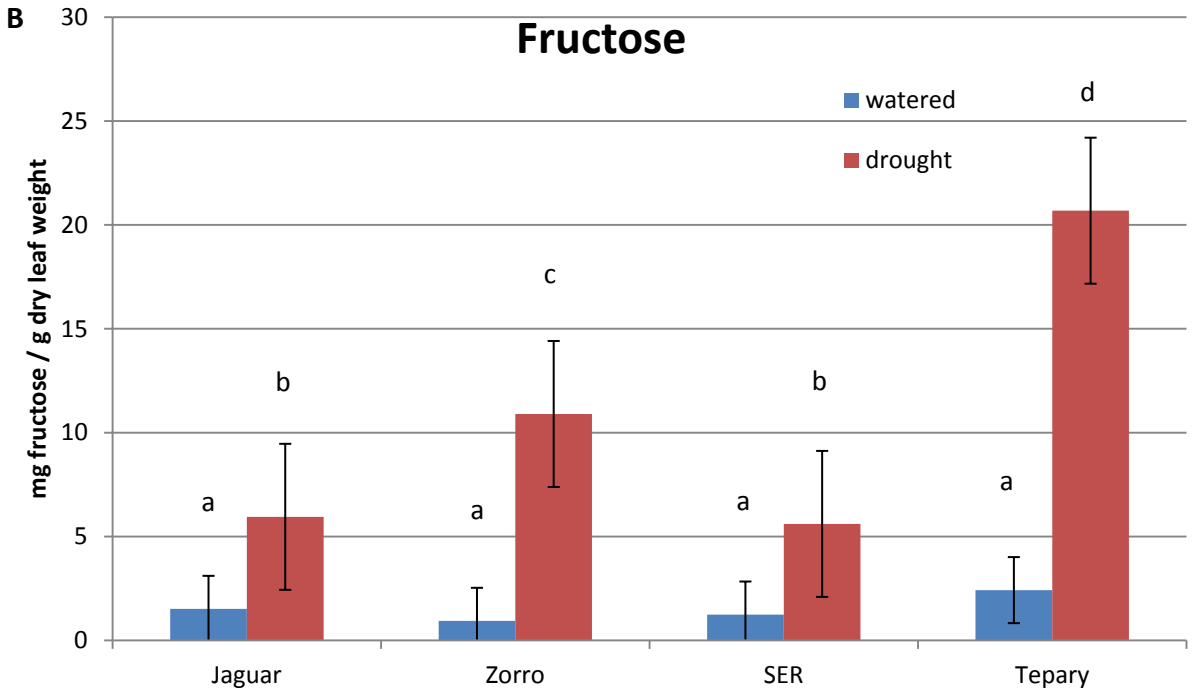
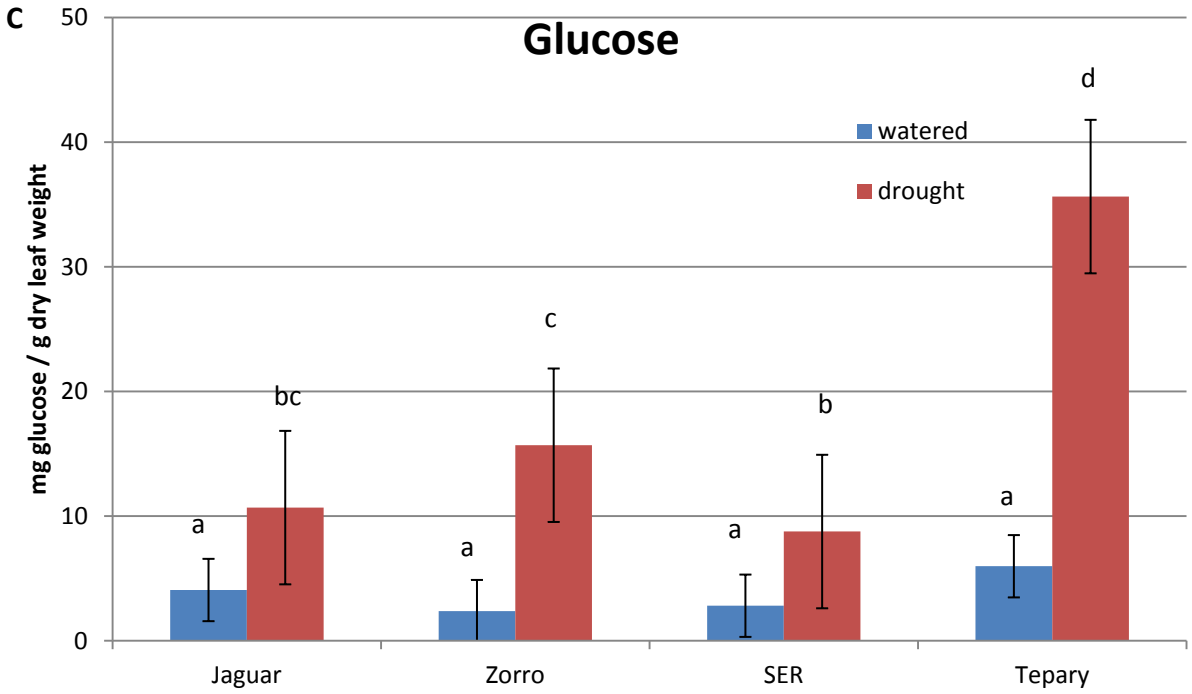


Figure 3 (cont'd)





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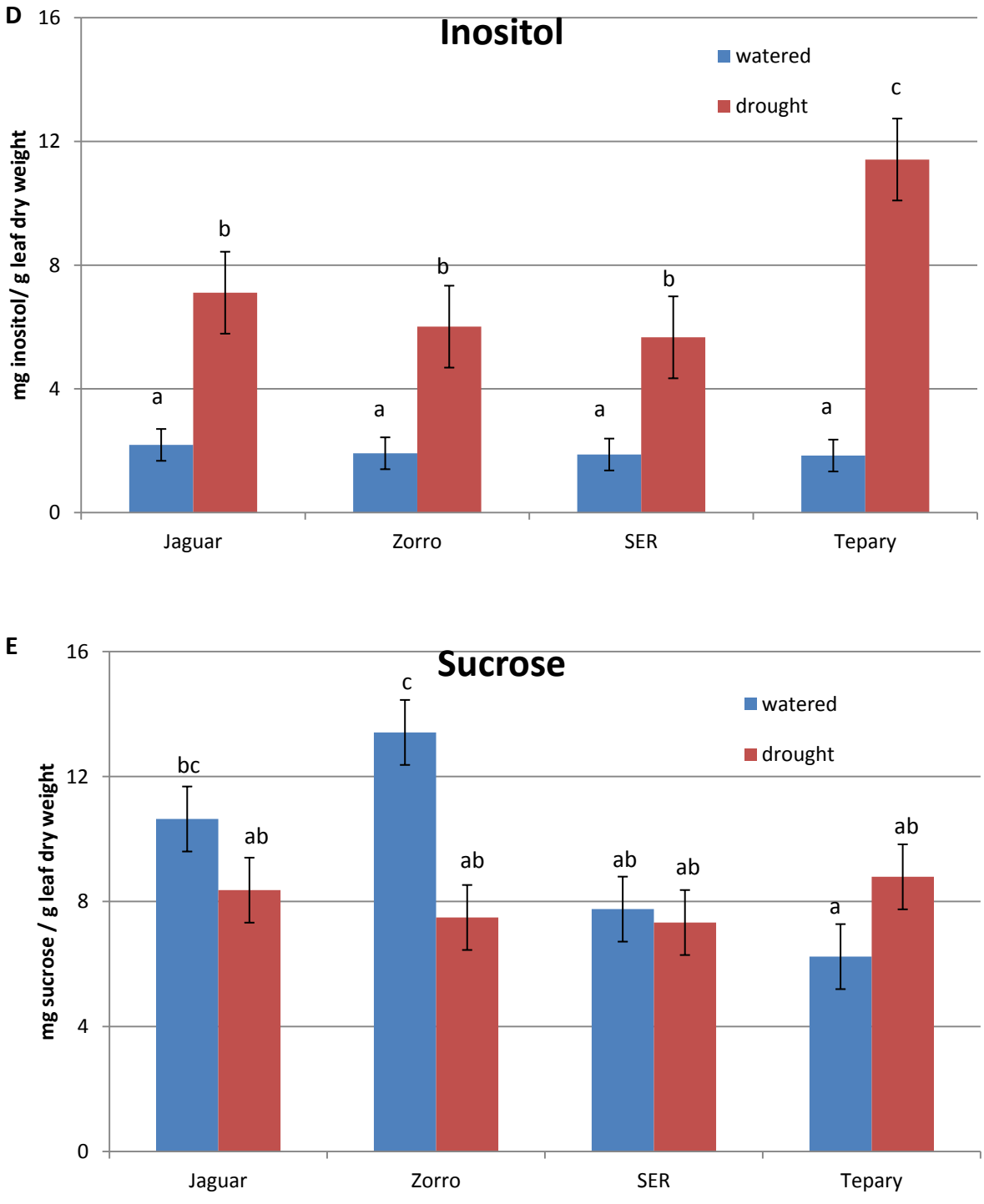

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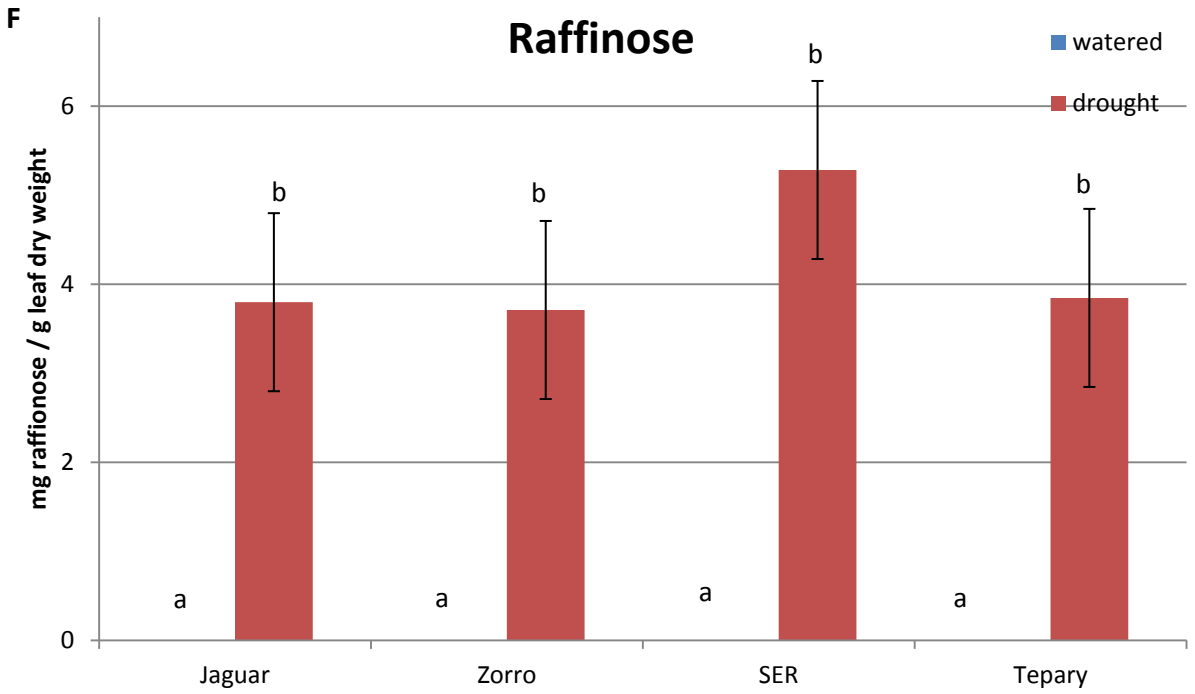
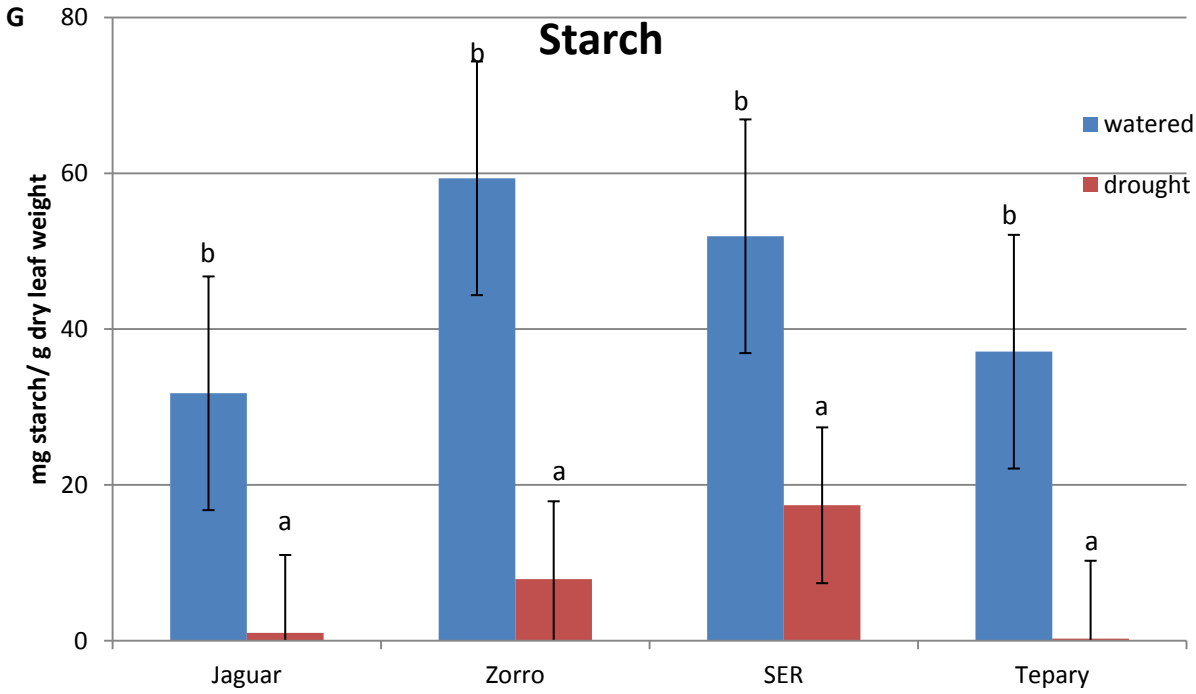




for all genotypes and treatments. Under drought stress, Zorro had the highest levels of glucose among the common bean lines, but tepary bean had an even higher concentration. Inositol overall had concentrations similar to fructose, and under drought stress, tepary had a higher concentration than the other genotypes (Figure 3D).

Levels of sucrose were similar between the well-watered and drought stress treatments; only genotype Zorro had a significant difference between the two treatments (Figure 3E). Conversely compared to other sugars, although the drought treatment had no differences among the four genotypes, the well-watered treatment had a narrow spectrum of differences among the genotypes, with tepary having the lowest concentration and Zorro having the greatest concentration. No raffinose was detected in any of the well-watered samples, but the drought treated genotypes all had similar amounts of raffinose in their leaves (Figure 3F). The concentration of raffinose was similar to that of malic acid and much lower than that of glucose or fructose.

Well-watered samples contained significantly greater concentrations of starch than drought treated samples. However, within a treatment, no differences in starch content could be detected among genotypes.

When comparing common bean genotypes tested in this experiment, all genotypes had the same level of carbohydrates under well-watered conditions, and they also had similar increases when exposed to drought stress. The tepary bean genotype, although similar to the common bean genotypes under well-watered conditions, had a significantly larger increase in a number of carbohydrates when exposed to drought stress.

Exogenous abscisic acid

As expected, increasing concentrations of exogenous ABA decreased the stomatal conductance of all bean lines that were tested (Figure 4). Not all lines showed similar decreases in conductance. Overall, tepary was the least responsive to ABA, with the smallest decrease in conductance relative to the no ABA treatment. However, tepary also had the lowest stomatal conductance under the control treatment. SER-16 was the most sensitive to ABA exposure, having the largest relative drop in stomatal conductance at low and medium concentrations of exogenous ABA. Bean lines Jaguar and Zorro were similarly sensitive to ABA and did not appreciably drop their stomatal conductance until moderate concentrations of ABA (5 mM) were applied. The greatest differences among lines were found at low to moderate concentrations of ABA (0.1 mM and 0.5 mM), where SER-16 is especially affected by the ABA exposure while Jaguar and Zorro were not affected as much. At 1 mM ABA, all lines had similarly low stomatal conductances.

Endogenous abscisic acid of leaf tissue and root tissue under drought

Under well-watered conditions, no significant differences in endogenous ABA levels were found for any of the bean lines tested, nor did mean ABA content differ on any of the days (Figure 5A). ABA concentrations were very low in all bean varieties under well-watered conditions. Under drought stress, the concentration of ABA increased by nearly two orders of magnitude (Figure 5B). Jaguar is the only line that was statistically different from the other lines in its concentration of ABA under drought stress: it accumulated more ABA in its leaves in response to drought stress than the other lines. After rewatering, ABA concentrations were reduced for all varieties. The ABA concentrations on days 4 and 5 after initiation of drought

Figure 4 - Exogenous abscisic acid
The stomatal conductance of four bean lines after exposure to several concentrations of exogenous abscisic acid. Bars represent standard error. Treatment means that share no letters are significantly different from each other (alpha = 0.05).

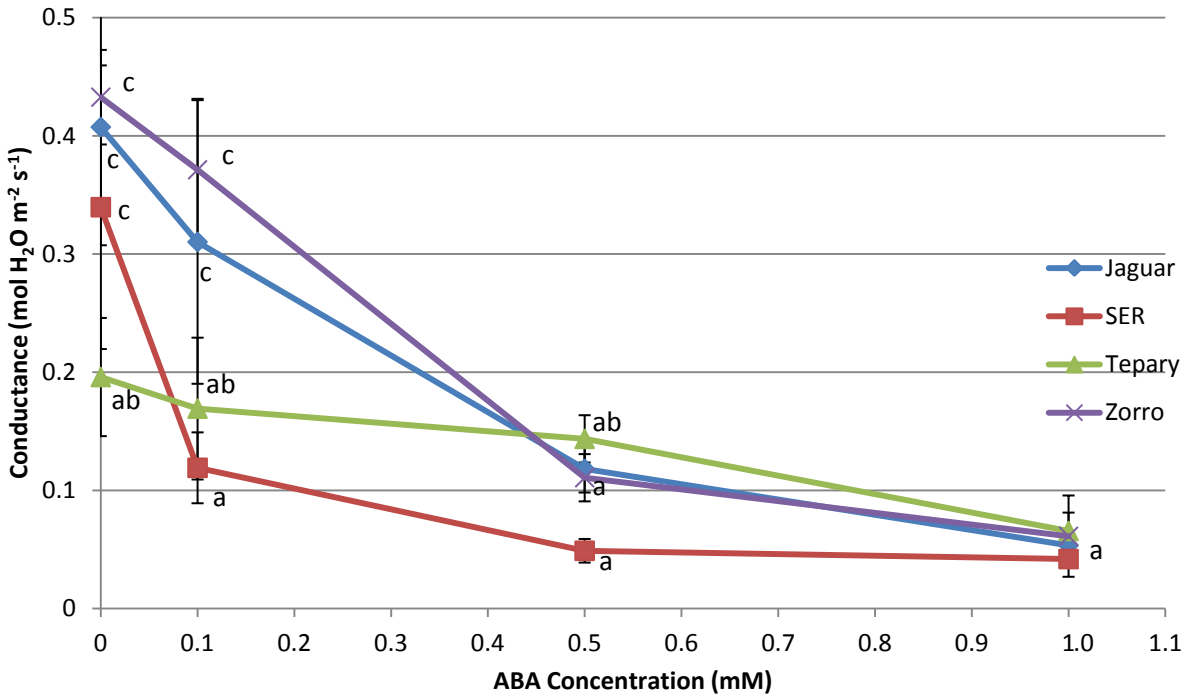


Figure 5 - Endogenous abscisic acid
The endogenous levels of abscisic acid in the leaf tissue of four different bean lines exposed to either (A) well-watered conditions or (B) drought stress and recovery. In (B), the drought treatment was started on day 0, and plants were rewatered and allowed to recover on days 3-5. Note the difference in scale in the y-axis between (A) and (B). Bars represent standard error. Treatment means that share no letters are significantly different from each other (alpha = 0.05).

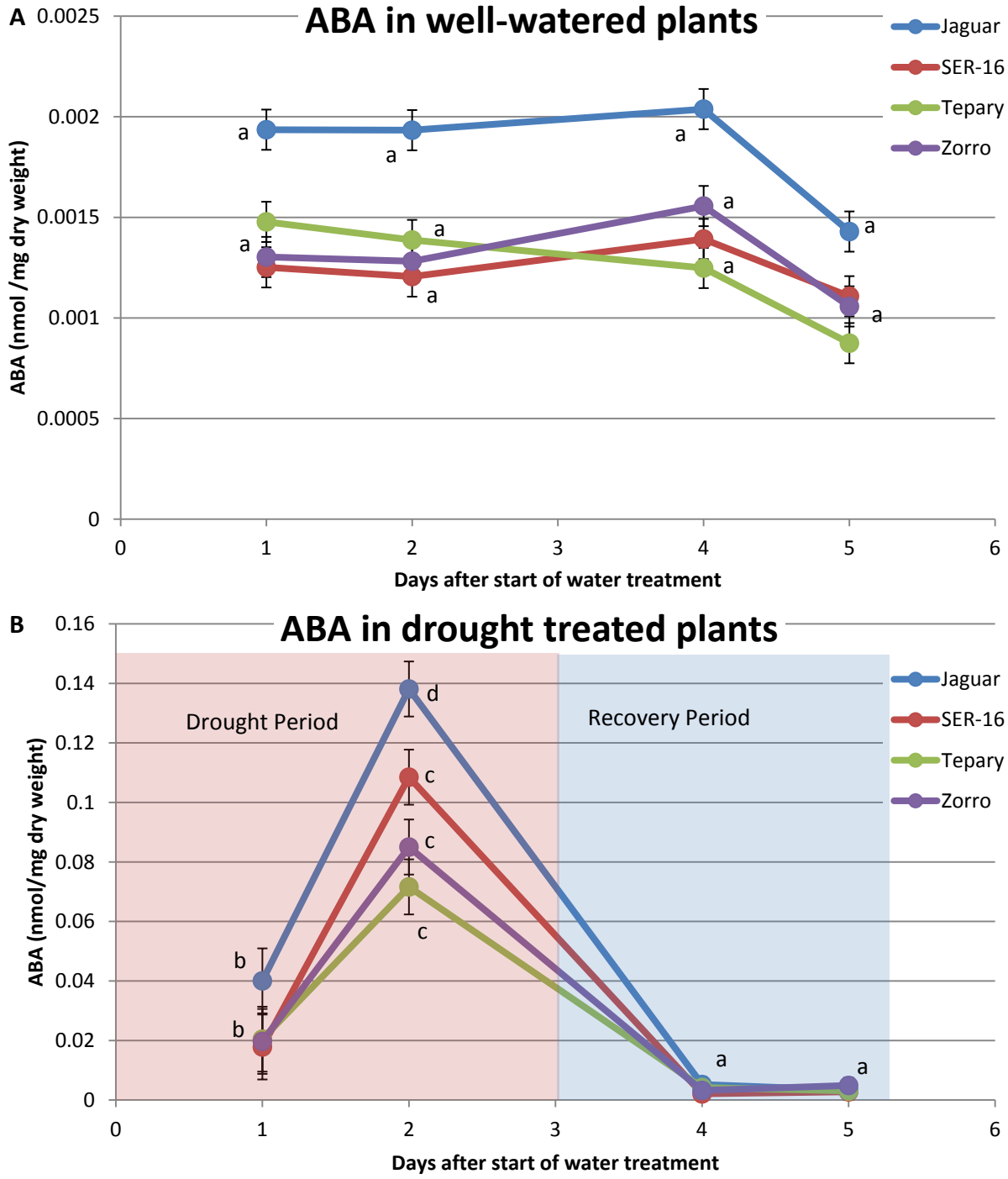
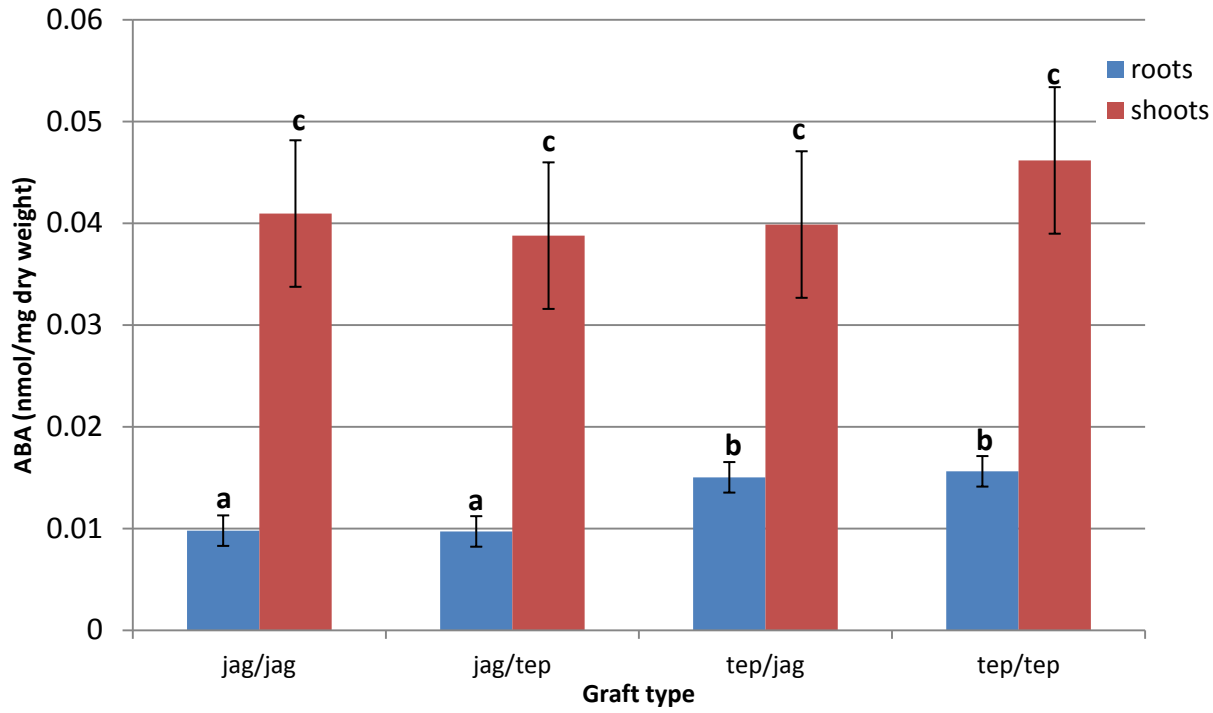


Figure 6 - Abscisic acid in roots and shoots
The concentration of abscisic acid in root tissues (blue bars) and shoot tissues (red bars) of different graft combinations between common bean genotype Jaguar and an improved tepary bean line. Tissues samples were taken during a period of moderate drought stress imposed by withholding water. Graft types are identified as shoot/root. Error bars represent standard error. Root concentrations not sharing a capital letter above them are significantly different from each other while shoot concentrations not sharing a lowercase letter are significantly different from each other (alpha=0.05).



treatment (1 and 2 days after rewatering, respectively) were statistically indistinguishable from the ABA concentrations of the well-watered controls.

Bean plants have lower concentrations of ABA in their roots compared to the leaf tissue concentrations (Figure 6). No significant differences were found among the different graft types for the concentration of ABA in leaf tissues. However, graft types tepary/Jaguar and tepary/tepary had significantly higher root concentrations of ABA than graft types Jaguar/Jaguar and Jaguar/tepary. The leaf ABA concentration in this experiment was similar to that found one day after the imposition of drought stress in the previous experiment (Figure 5B). This level indicates a moderate level of drought stress, and for both experiments, no varietal differences in leaf ABA concentration were detected at this level of drought stress.

Discussion

Proline and carbohydrates

When considering levels of free proline in bean leaves, no significant differences could be detected between well-watered bean plants and those experiencing drought stress. Proline content thus apparently plays no role in common beans during moderate drought stress events; because its levels do not change, proline is unlikely to mitigate the stress that beans experience nor are proline levels perturbed as a downstream effect of drought stress. These results are similar to previously reported effects of moderate drought stress on proline levels in common beans (Rosales et al., 2012). While some xerophytes use free proline accumulation in shoot tissues to mitigate severe drought stress (Hoekstra et al., 2001), this same mechanism seems unlikely to play a role in common beans.

Unlike free proline, many of the carbohydrates studied in this experiment had a significant and consistent response to drought stress. Although proline and many carbohydrates were present in comparable quantities under well-watered conditions, several soluble carbohydrates had dramatic increases in concentration under drought stress. Although too imprecise and expensive to use as a main analysis, the preliminary metabolite screen was especially useful for identifying compounds that could be analyzed in more detail in the soluble carbohydrates study.

When comparing only common bean genotypes, very few differences were found amongst them for the accumulation of carbohydrates under drought stress. Zorro accumulated significantly more of the hexoses, glucose and fructose, under drought stress than at least one of the other common bean genotypes. Zorro is more stress tolerant than Jaguar (Kelly et al., 2009*a*), so its greater concentration may be an adaptive mechanism to stress. However, this increase in concentration is not seen in SER-16, which is a genotype with greater drought tolerance than Zorro (Beebe et al., 2008; Kelly et al., 2009a). This lack of the mechanism may be explained by SER-16 having other mechanisms of drought tolerance that have a greater impact on yield under stress, so it can lack high concentrations of hexoses under drought stress but still perform better than Zorro. Zorro and Jaguar belong to the same market class and are genetically close to each other, so it is possible that Zorro's higher concentration of these compounds gives it the advantage in terms of performance under drought stress.

When tepary bean is compared to the common bean genotypes, it shows a much greater accumulation of the hexoses glucose and fructose as well as the cyclic sugar alcohol inositol, a direct derivative of glucose 6 phosphate. Tepary's higher concentration of these compounds in its

leaf tissues under drought stress could contribute to its greater drought tolerance when compared to common bean genotypes. Using the form of the van 't Hoff equation:

$$\Psi_\pi = -MiRT \qquad \text{(Equation 1)}$$

where Ψ_π is the osmotic potential, M is molar concentration of the solute, *i* is the van 't Hoff dissociation factor, R is the ideal gas constant, and T is the absolute temperature, the approximate contribution of the soluble carbohydrates to osmotic adjustment in tepary under stress is -0.1 MPa compared to approximately -0.05 MPa for the common bean genotypes. This difference in osmotic adjustment is due to tepary's higher concentrations of glucose, fructose, and inositol. The accumulation of these compounds and the ensuing decrease in leaf water potential could allow tepary to delay wilting and continuing growing under water-limited conditions.

Relevant to all genotypes in this experiment, inositol conjugates with UDP-D-galactose to form a precursor of raffinose biosynthesis (Loewus and Murthy, 2000), so the increase seen in inositol under drought stress in all genotypes may be related to their higher concentrations of raffinose under drought stress. Raffinose was undetectable in bean leaf tissues under well-watered conditions, but drought stress greatly increased the raffinose content in all genotypes. Raffinose accumulation in response to abiotic stress is seen in a number of species (Barchet et al., 2014; Richter et al., 2015; Wenzel et al., 2015), and it possibly acts as an osmoprotectant to protect against both osmotic and oxidative stress (Nishizawa et al., 2008).

Moderate drought stress in bean plants led to a decrease in the concentration of starch in leaf tissue. A decrease in starch in response to drought stress is seen in beans and in other plant

species and most likely results from an inhibition of synthesis (Vassey and Sharkey, 1989; Geigenberger et al., 1997; Escobar-Gutierrez et al., 1998). Well-watered Zorro leaves had higher concentrations of sucrose than the leaves of other genotype and treatment combinations, but no other physiologically significant differences in sucrose concentration were observed among treatment means. Sucrose's stability is remarkable not only because a decrease in starch synthesis could shunt glyceraldehyde-3-phosphate towards sucrose synthesis but also because the increase in fructose and glucose concentrations could result from the breakdown of sucrose by invertase or sucrose synthase. Under drought stress, the sucrose pool could be a sensitive regulator of reduced photosynthate from stress, reduced starch synthesis, and increased fructose and glucose pools. Indeed, sucrose regulates the expression of a number of genes independent of fructose and glucose (Rolland et al., 2006), so any increase or decrease in the size of the sucrose pool could trigger negative-feedback loops that return sucrose to normal values.

Malic acid was seen in higher concentrations in the leaves of beans exposed to drought stress. Malic acid pools increased in response to hypoxia in moss and heavy metal exposure in *Silene cucubalus* (Bailey et al., 2003; Rut et al., 2010) and decreased in response to chemically induced oxidative stress and a combination of heat and drought stress in Arabidopsis (Koussevitzky et al., 2008; Obata et al., 2011) . Interestingly, NADP-malic enzyme increases in activity under drought or osmotic stress in many C_3 plants (Liu et al., 2007; Doubnerová and Ryšlavá, 2011). NADP-malic enzyme converts malate and $NADP^+$ to pyruvate, CO_2, and NADPH. The increase in malic acid, especially if it took place in the chloroplasts of leaf cells, could play a small role in pH related stress signaling (Edwards et al., 1998). Indeed, one paralog of NADP-malic enzyme in Arabidopsis functions in the chloroplast (Wheeler et al., 2005). NADP-malic enzyme increases in content and activity in response to ultraviolet B stress (Casati

et al., 1999), and in a study of three bean genotypes, NADP-malic enzyme content was positively correlated with tolerance to ultraviolet B stress (Pinto et al., 1999). In tests of several barley genotypes, only the drought tolerant genotypes upregulated the expression of NADP-malic enzyme in response to drought stress (Guo et al., 2009). However, NADP-malic enzyme breaks malic acid down. What about its synthesis? In Arabidopsis, drought stress significantly increases the expression of fumarase, the enzyme responsible for the production of malic acid from fumarate, malic acid's direct precursor, but has little to no effect on the expression of other genes in the tricarboxylic acid cycle (Kilian et al., 2007; Winter et al., 2007). Additionally, in maize roots exposed to stress, the flux of carbon to malic acid via PEP carboxylase is an order of magnitude greater than the flow of carbon out of malic acid via NADP malic enzyme (Edwards et al., 1998). The increase in malic acid concentration seen in beans under drought stress could allow them to regulate cellular pH and produce reducing power and CO_2 via the NADP-malic enzyme pathway, with the possibility that the CO_2 released could be refixed in the chloroplast. Further, malic acid could act as a counter-ion to potassium and thus aid in the regulation of stomatal closure.

Abscisic acid

Different bean genotypes have different stomatal sensitivities to ABA (Figure 4). This sensitivity is not correlated with a genotype's drought tolerance. While the drought tolerant tepary bean is comparatively insensitive to ABA, the drought tolerant common bean genotype SER-16 is more sensitive than any other genotype tested. These differential sensitivities could indicate a divergence in the drought stress strategies of these two bean genotypes; despite both being drought tolerant, tepary bean and SER-16 belong to different species, so the genetic

distance between them could have allowed different stress adaptation strategies to evolve in each one. SER-16 has a highly responsive strategy that regulates metabolism at early or smaller stress signals while tepary has a more conservative strategy that keeps primary productivity low under all conditions with less reliance on sensitivity to stress signals for survival. Differences in sensitivity to ABA among *Phaseolus* genotypes is a new finding, and future studies should include additional genotypes not used in this study to test if the patterns of sensitivity found in this experiment hold true for the wider bean germplasm.

Leaf ABA levels rose sharply in bean plants as drought intensified, but they remained low and stable under well-watered conditions (Figure 5). Varietal differences in ABA concentration only appeared under severe stress; drought susceptible common bean Jaguar accumulated more ABA in its leaves than any other genotype. While ABA is necessary to activate many stress protective responses, the hyperaccumulation of ABA in Jaguar could indicate a disruption of signaling that is coincident with a harmful, unregulated stress response (Seiler et al., 2011; Sreenivasulu et al., 2012). Tepary bean, the most drought tolerant of the lines tested, had the lowest concentrations of ABA in its leaves during the most severe stress period, and its low concentration could indicate a well regulated ABA signal that is an advantage to survival and reproduction under stress conditions. Thus, as the bean plants transition from moderate to severe drought stress, the ABA levels of tolerant and susceptible genotypes diverge as the former maintain ABA homeostasis while the latter has its drought signaling disrupted (Sreenivasulu et al., 2012).

When comparing the bean lines' sensitivities to ABA (Figure 4) and endogenous levels of ABA under stress (Figure 5B), we gain a greater insight into their stress response than from either of those facets alone. The extremely drought tolerant tepary is neither as sensitive to ABA

nor does it produce as much under severe stress, suggesting a strong constitutive mechanism of protection less reliant on stress signaling. The drought tolerant SER-16 has both high levels of ABA under stress and is very sensitive to ABA exposure, suggesting a more reactive stress response strategy. Jaguar, a susceptible genotype, and Zorro, a moderately tolerant genotype, both have the same sensitivities to ABA, but Jaguar produces more ABA under severe drought stress. Zorro's ability to maintain ABA homeostasis under severe stress could be why it has an advantage over Jaguar under stress conditions despite sharing its sensitivity to ABA.

As a whole, on a dry plant tissue basis, leaves produce more ABA than roots in drought stressed bean plants (Figure 6). These results correlate with previous studies that find shoot tissue the site of ABA production (Holbrook et al., 2002; Christmann et al., 2007). For shoot ABA concentrations, no differences were found among any of the graft types, and these results matched previous results about shoot ABA levels under moderate stress (Figure 5). However, root ABA concentration significantly differed among graft types: tepary/Jaguar and tepary/tepary had higher ABA concentrations in their roots than Jaguar/Jaguar and Jaguar/tepary. Given that the graft types with higher root ABA shared their shoot identity but had different root identities, shoot identity appears to determine ABA concentrations in root tissues. Interestingly, the drought tolerant tepary bean had a higher concentration of root ABA than the drought susceptible common bean genotype Jaguar, but further bean genotypes' root ABA levels will need testing to determine if high root ABA correlates with drought tolerance in field settings. Mechanically, this correlation is plausible because ABA can influence root hydraulic conductivity (Thompson et al., 2007; Kudoyarova et al., 2011) and change the characteristics of water supply from the roots.

In Figure 5B, two days after the imposition of stress and when drought stress was severe, ABA concentrations in leaves reached their highest point as well as the point at which

appreciable differences in ABA among cultivars appeared. The ABA levels in the shoot tissue in Figure 6 were very close to the ABA levels of leaves one day after the imposition of stress, and drought stress was only moderate at that point. Using these ABA concentrations as a reference, as drought stress intensified, genotypic differences in root ABA appeared sooner than genotypic differences in shoot ABA. Tepary sent a greater initial stress signal to its roots than Jaguar did, and the metabolic changes this signal induced could have better prepared tepary for upcoming stress and prevented a larger stress signal in the shoot when stress did arrive.

Conclusions

Free proline plays little to no role in stress protection or response in common and tepary beans, but the concentrations of certain carbohydrates and organic acids do. Tepary's higher concentration of some of these carbohydrates likely contributes to its greater drought tolerance. Future studies should look at the subcellular localization of some of the compounds studied, like malic acid, glucose, fructose, and raffinose, as well as the activity of the NADP-malic enzyme, in tepary and common bean to investigate any differences and further define the function of these compounds in stress response. Analyzing the concentrations of glucose and fructose in drought stressed leaf tissue could aid in screening for drought tolerant genotypes, but tolerant genotypes lacking this mechanism would be undetected, and the time and specialized equipment required would limit its application. Both within and among species in *Phaseolus*, variation exists for sensitivity to ABA and concentrations of ABA produced under stress, but neither measurement alone correlates with a variety's drought tolerance. However, combining information about sensitivity to ABA and endogenous levels of ABA allows for a more informative picture of a

genotype's stress response. Shoots are responsible for ABA production and its concentration in the roots of bean plants. Additional studies on root ABA concentration in stressed bean plants in a wider range of germplasm would help determine if correlations exist between root ABA and drought tolerance.

Chapter 3: Drought stress and photosynthesis

Introduction

Common beans (*Phaseolus vulgaris* L.) are a staple source of protein and nutrients in many parts of the world, especially Central America and East Africa (Cavalieri et al., 2011). Drought stress limits the yield of common beans in roughly 60% of the regions in which it is produced in any given year (McClean et al., 2011). Breeding bean cultivars with improved drought tolerance is thus crucial to the stability of the global bean crop (Beebe et al., 2012), which in turn supports the food security and economic well-being of the farmers producing beans. To assist breeders in developing more tolerant cultivars, the physiology of drought tolerance in common beans should be investigated further (Beebe et al., 2013). While research in the drought tolerance mechanisms of other crops (Cattivelli et al., 2008; Passioura, 2012) helps inform efforts in beans, ultimately the presence and range of these mechanisms in common bean germplasm must be investigated before they can be used in a breeding program. Furthermore, there is a need for drought studies that integrate several different types of metabolic and physiological measurements (Pinheiro and Chaves, 2011). The present study investigates several physiological traits and their response to drought stress in a small group of bean genotypes contrasting for drought tolerance.

Water stress impacts a plant's productivity by decreasing its rate of photosynthesis. Considerable debate still exists about the relation between photosynthesis and agronomic yield (Long et al., 2006), but measurements of photosynthesis still offer rough insight into the integrated metabolism of a plant and how water stress affects that (Chaves, 1991). The sensitivity of reproductive tissues to drought stress creates a disconnect between photosynthesis and agronomic yield. Drought stress causes the abortion of developing reproductive tissue. Tolerant

genotypes in an agronomic setting have lower fruit abortion rates, often by remobilizing photosynthate reserves from other plant parts (Araus et al., 2002; Yang and Zhang, 2006; Tolk et al., 2013). While much of the previous research was done on cereals, tolerant common bean genotypes also have lower pod abortion rates (Boutraa and Sanders, 2001; Lizana et al., 2006). Additionally, comparisons of photosynthesis within a species avoid the variance attendant with broader studies and allow meaningful correlations to be revealed (Fischer et al., 1998). Closely linked to photosynthesis is stomatal conductance, how open a leaf's stomata are to the influx of carbon dioxide and the efflux of water vapor. Plants must balance their carbon and water resources. Under drought stress, plants close their stomata to conserve water, but this also reduces the supply of carbon dioxide available for photosynthesis. Closure of stomata is the main limiting mechanism of photosynthesis under drought stress (Flexas et al., 2004). The balance a plant strikes between carbon gain and water loss determines the degree of its drought tolerance (Pinheiro et al., 2005).

Tepary beans (*Phaseolus acutifolius* A. Gray) are a drought tolerant species native to the Sonoran desert and used as an arid land crop within Northwest Mexico and the American Southwest for hundreds of years (Nabhan and Felger, 1978). Tepary is closely related to common beans, and with embryo rescue, tepary and common bean can be hybridized with each other (Mejía-Jiménez et al., 1994; Araújo et al., 2014). A handful of studies compared relative responses of common beans and tepary beans subjected to heat and drought stress (Markhart, 1985; Castonguay and Markhart, 1991, 1992; Sanders and Markhart, 1992; Udomprasert et al., 1995), but research into tepary waned for some years until tepary's potential as a genetic resource for improving common beans was more fully utilized (Butare et al., 2011; CGIAR, 2015). In

addition to its useful genetics, tepary's physiological traits are a model for which traits to alter in common beans to improve their abiotic stress tolerance (Rao et al., 2013).

To investigate drought response, the current research studied physiological traits within common bean and tepary beans and how these two species compared with each other. Drought's effects on photosynthesis in beans were a particular focus and were measured using gas exchange, chlorophyll fluorescence, and grafting. Stress and its relation to morphology was also investigated by looking at leaf traits and the abscission of reproductive tissues.

Materials and methods

Rates of pod abscission under drought stress

The common bean varieties Fuji and Zorro were sown in 1 L square, black plastic pots and grown in a growth chamber set to a constant 22° C, 12 h light and 12 h dark cycle, and a light intensity of 200 µmol photons m^{-2} s^{-1}. Plants were grown in Suremix Pearlite potting media (Michigan Grower Products, Galesburg, MI, USA) with 32 replications per variety. Fuji is a cultivar within the specialty Otebo bean market class that has substantially lower yields under drought stress (Kelly et al., 2009*b*). Zorro is an elite cultivar within the black bean market class that is moderately tolerant to drought stress and generally yields well (Kelly et al., 2009*a*). After sowing, the plants were regularly watered with a half-strength Hoagland nutrient solution. Once the unifoliate leaves were fully expanded, all plants were placed on a limited watering regime: every two days, each pot was only given enough water to bring it up to 30% of pot's total water capacity, as determined by weighing on a scale. A month after sowing, the plants began to flower. Two weeks after flowering, the number of pods and trifoliate leaves on each plant was

Table 1 - Bean genotypes used in experiments this chapter
The table below contains brief descriptions of the bean genotypes used by the experiments that are discussed in this chapter.

Name	Description	Level of drought tolerance
Big Fields Brown	Pa, landrace	Very tolerant
Black Tepary	Pa, landrace	Very tolerant
Fuji	Pv, otebo bean	Susceptible
Jaguar	Pv, black bean	Susceptible
RAB-651	Pv, small red bean bred by CIAT	Tolerant
Sacaton White	Pa, landrace	Very tolerant
San Ignacio	Pa, landrace	Very tolerant
SER-16	Pv, small red bean bred by CIAT	Tolerant
SER-95	Pv, small red bean bred by CIAT	Tolerant
TB1 tepary	Pa, improved tepary line bred by Dr. Tim Porch	Very tolerant
Tohono O'dham	Pa, landrace	Very tolerant
Tucson Brown	Pa, landrace	Very tolerant
Wild tepary - Chihuahua	Pa, wild accession	Very tolerant
Wild tepary - Tiburón Island	Pa, wild accession	Very tolerant
Zorro	Pv, elite black bean	Moderately tolerant

Legend: Pv - *Phaseolus vulgaris*, Pa - *Phaseolus acutifolius*

counted, and two weeks after that first counting, the number of pods and leaves on each plant was counted again. Pods of any size or developmental stage were included in the count, but only fully expanded trifoliate leaves were included in the count. Varietal and time means were compared using PROC MIXED in SAS version 9.4 (SAS Institute, Cary, NC, USA).

Chlorophyll fluorescence of young stressed bean leaves

The plants were grown as described in the 'Rates of pod abscission under drought stress' section above with the following modifications. Four common bean genotypes were used: Jaguar, RAB-651, SER-16, and Zorro. Jaguar is an older black bean variety whose susceptibility to biotic and abiotic stresses led to its replacement by Zorro. SER-16 and RAB-651 are two small red bean lines bred in Colombia by CIAT as part of their development of abiotic stress tolerant lines, the latter bred for tolerance to low phosphorous, and both have shown tolerance to stress in field settings (Beebe et al., 2008, 2013). Plants were split into well watered and drought stressed treatments with six replications of each genotype per treatment. The experiment was arranged as a randomized complete block design. After the first trifoliate leaves fully matured, the watered treatment continued to receive daily watering with deionized water while the drought treatment received no additional water.

Three days after the start of differential watering, measurements of maximal photosynthetic efficiency (Fv/Fm) were made on the leaves that were dark-adapted over night. Measurements were made at the end of the dark period in the dark (a flashlight was used to provide illumination for the work) to avoid disrupting dark adaptation. The measurements were made with the fluorescence head of the LI-COR 6400XT (LI-COR Biosciences, Lincoln, NE, USA). After the Fv/Fm measurements, the chamber lights turned on and the plants adapted to the

light for two hours. Then, the LI-COR's chlorophyll fluorescence head was again used to measure photosystem II efficiency (Φ_{PSII}). All plants were then rewatered, and after one week, the aboveground tissues of the plants were harvested, dried in a 60° C oven, and the final dry weight of each plant measured. Data were statistically analyzed and treatment means compared using PROC MIXED in SAS 9.4. An alpha of 0.05 was used as the threshold for significance. When a treatment or interaction was not significant according to the analysis of variance (ANOVA), the individual means within that treatment or interaction were compared using the much more conservative Tukey's adjustment.

Leaf water potential of stressed plants

This experiment was grown under the same conditions as described above in the 'Rates of pod abscission under drought stress' section but with the following modifications. Four genotypes were planted: Jaguar, SER-16, Zorro, and a drought tolerant tepary bean line TB1 provided by Dr. Timothy Porch from USDA-ARS in Mayaguez, Puerto Rico. Plants were grown in a growth chamber with the temperature set to 25°C during the light period and 20° C during the dark period and a 14 h light and 10 h dark cycle. Light intensity (PAR) was 400 µmol photons m^{-2} s^{-1}. Plants were grown in 10 L black plastic pots in a common pot setup: one plant of each genotype per pot. Twelve pots arranged in a randomized complete block design were grown in total. Four weeks after germination, the third trifoliate leaves of the common beans had fully expanded, and the pots were watered to pot capacity. Then, water was withheld for three days, and the plants appeared drought stressed by the end of this period. In the midmorning of the third day after watering stopped, the latest fully expanded trifoliate of each plant was cut off at the base of the petiole, the leaves sealed in plastic bags, and the bags kept in a cool, dark container

until their analysis. A pressure bomb (PMS Instruments, Albany, OR, USA) was used to measure the leaf water potential of each leaf. The leaves were sealed in the pressure bomb with the cut petiole sticking out, and nitrogen from a compressed gas tank was used to increase pressure in the chamber until water returned to the cut surface of the petiole, at which point the pressure reading was recorded as the leaf's water potential.

A-C_i curves in well-watered conditions

This experiment was performed as the leaf water potential experiment described above but with the following differences. The four genotypes planted were Jaguar, Tepary, Zorro, and SER-95, a small red variety bred for drought tolerance by CIAT. The plants were grown individually in 5 L plastic pots with four to six replications per variety. The temperature regime was 28° C during the light period and 20°C during the dark period. Four weeks after sowing, the third trifoliate leaves were fully expanded, and measurements of the photosynthetic rate at different intercellular CO_2 concentrations (A-C_i) commenced. The plants were kept well-watered for the duration of the experiment.

For three consecutive days, A-C_i measurements were made from mid morning to mid afternoon, measuring two replications per day using the autoprogram feature of the LI-COR 6400XT. Six replications of each genotype were measured in total. In addition to the limited time period during which measurements took place, time of day effects were also controlled by randomizing within a replication the order in which genotypes were measured. An entire block of plants, consisting of one of each genotype, was measured before moving on to the next block. The reference CO_2 concentration started at 1500 µmol mol^{-1} and decreased to 1250, 1000, 800, 600, 500, 400, 300, 200, 150, 100, 50, and finally 0. Measurements were automatically taken

when the stability criteria involving the rate of change of photosynthesis and stomatal conductance were met; to measure the A-C_i curve of one plant took approximately 25 minutes. The entire measurement period took place under a saturating light intensity of 1000 µmol photons m^{-2} s^{-1}. Afterwards, the measured data were analyzed using a spreadsheet to determine the maximum carboxylation efficiency of rubisco (V_{cmax}), electron transport rate (J), triose phosphate use (TPU), and mesophyll conductance (g_m) (Sharkey et al., 2007). The day respiration rate (R_d) was fixed at 0.66 µmol m^{-2} s^{-1} (at 25° C), and calculations were done with a Γ^* of 3.97 Pa (at 25°C); the R_d and Γ^* values come from Sean Weise's experimental determination of these parameters in soybean (unpublished data).

The values of V_{cmax}, J, TPU, and g_m were analyzed in SAS 9.4 using the PROC MIXED statement as a randomized complete block design experiment with replication as the blocking factor. For ANOVAs that were significant, means were separated using the least significant difference and an alpha of 0.05.

Gas exchange measurements over increasing drought stress

These experiments used the same bean genotypes, common pot setup, and environmental conditions described in the 'Leaf water potential of stressed plants' section above but with the following modifications. The pots in which plants were growing were split into two treatments: a well-watered control and a drought stress treatment, each with six replications. All treatments were kept well-watered until the third trifoliate leaf was fully expanded. Then, the LI-COR 6400XT was used to take gas exchange measurements on the latest fully expanded trifoliate leaf while the plants of both treatments were still well watered. Gas exchange measurements were taken under a light intensity of 1000 µmol photons m^{-2} s^{-1}, a CO_2 concentration of 400 µmol mol^{-}

[1], uncontrolled relative humidity ranging from 54-59%, and a block temperature that ranged from 27-31 °C. Watering then stopped on the plants in the drought stress treatment while the well-watered treatment continued to receive water daily. Gas exchange measurements were taken daily for the next five days as stress in the drought treatment increased. On the sixth day, all treatments were rewatered to pot capacity, and the drought treatment was again allowed to dry down from days seven through ten. At midmorning on day ten, the latest fully expanded leaf was cut from each plant, and the leaf water potential of these leaves were measured according to the procedure described above in the leaf water potential experiment.

This experiment was replicated, and after watering was stopped on the drought treatment, the weight of each pot was measured using a high capacity electronic scale before gas exchange measurements were taken. Separately, the weight of individual empty pots, pots filled with dry soil mix, and pots filled with fully saturated soil mix were weighed. From these measurements, the maximum water holding capacity of the 10 L pots was determined as well as the percentage of maximum capacity each pot was at during the stress period.

Survey of tepary varieties

Eight different tepary landrace and wild varieties, kindly donated by Native Seeds (Native Seeds/SEARCH, Tucson, AZ, USA) for experimental purposes: Black Tepary, San Ignacio, Tucson Brown, Tohono O'dham, Big Fields Brown, Sacaton White, wild tepary collected from the Chihuahuan Desert, and wild tepary collected from Tiburón Island, Mexico. The common bean Zorro was included as a check. The improved TB1 tepary line was not included in this experiment because of space limitations, but it was grown with Zorro in the experiment 'Gas exchange measurements over increasing drought stress' detailed above, so rough

comparisons via comparative performance to Zorro should be possible. Previous work revealed that germination was a problem, so all seeds were scarified with a file, placed on moist filter paper in petri dishes, and kept in a 30° C environment. After three days, the majority of seeds had germinated, and they were transplanted to 10 L pots. The seeds were grown according to a randomized incomplete block design; four different genotypes were planted in each pot, and each of the nine genotypes used were replicated three times for a total of seven pots. The plants were then grown under the same conditions as described in the above section 'Leaf water potential of stressed plants'. When the third trifoliate leaf was fully expanded, water was withheld from all pots. Gas exchange measurements were taken one day and three days after the cessation of watering, representing mild and severe drought stress, respectively. Five days later, watering was reintroduced, and three days after watering was reintroduced, gas exchange measurements were taken again. Gas exchange measurements were taken according to the procedure described above in the gas exchange measurements over increasing drought stress experiment.

The experiment was analyzed in SAS as a randomized incomplete block design using the PROC MIXED procedure. As the ANOVA from the PROC MIXED procedure was significant for both genotype and stress level, Fischer's Least Significant Difference (LSD) was used to compare means.

Gas exchange of grafted beans

In order to examine the relative contributions of root tissue and shoot tissue to drought response, grafting experiments were performed between two contrasting genotypes: drought susceptible Jaguar and drought tolerant tepary. A flat each of Jaguar seeds and tepary seeds were planted and germinated in a growth chamber. One day after full germination, the plants

underwent grafting. The grafting procedure involved using a razor blade to cut the seedling in two at the stem 2 cm below the shriveled but still attached cotyledons. This was done for a second plant as well, yielding two shoots and two rootstocks. One shoot's stem end was pared on two sides to form a pointed wedge, and then a vertical cut was placed 1 cm deep into the top of the second rootstock's stem. The shoot wedge was inserted into the rootstock stem's cut so that the cut sides of both were in full contact with each other, and the entire graft junction was then wrapped in a small piece of Parafilm. The same was done to the remaining shoot and rootstock. A bag was placed over each plant to maintain a humid environment, and the plants were kept well-watered in the growth chamber. Grafts were performed in four combinations: Jaguar shoot grafted onto Jaguar rootstock, Jaguar shoot grafted onto tepary root, tepary shoot on Jaguar root, and tepary shoot on tepary root. Ten days after grafting, the bags were removed, and plants were transferred to 10 L common pots with one of each graft type in the same pot, eight of these common pots total. The plants were then grown normally under the environmental conditions described in the leaf water potential experiment described above.

After one month of recovery and growth, all plants were watered to pot capacity, and then all watering ceased. Gas exchange measurements were taken daily in midmorning for the five days after the cessation of watering as the pots gradually dried down. Gas exchange measurements were taken according to the procedure described above in the 'Gas exchange measurements over increasing drought stress' section. Intrinsic water use efficiencies were calculated by dividing each measurement's photosynthetic rate by its stomatal conductance.

Gas exchange data was analyzed as a repeated measures experiment in the statistical program SAS using the PROC MIXED procedure and the "repeated" statement. The resulting

ANOVAs found both graft type and genotype significantly affected the dependent variables tested, so individual means were compared using LSD.

Leaf density and stomatal density

Jaguar and tepary were examined for density of leaf tissue and the density of stomata on the abaxial surface of the leaves. These two genotypes were chosen because they are genetically distinct and differ in drought tolerance. Fifteen replications of each genotype were planted, and plants were grown individually in 5 L plastic pots. Otherwise, the plants were grown in the same environmental conditions as described above in the 'Leaf water potential of stressed plants' section. After the third trifoliate leaf was fully expanded, the plants were all exposed to moderate drought stress by withholding watering for four days and letting the pots dry down before continuing watering. The plants were grown normally for two more weeks to allow leaves that were expanding during the drought period to mature, and then leaflets from these leaves were excised to obtain a whole leaf blade with no petiole or petiolule.

Immediately after a leaflet was detached, it was weighed on an electronic balance and was photographed. The resulting photograph was analyzed using the software Easy Leaf Area (Easlon and Bloom, 2014) to obtain the surface area of the leaflet, and density was calculated from weight and area.

Other detached leaflets were used to determine stomatal density with four replications per genotype. A thin layer of clear nail polish was applied to the abaxial surface of a leaf. After the nail polish dried, forceps were used to peel off and remove a small patch of the dried polish, and this thin film of dried polish was examined under a microscope, revealing a relief of the abaxial

leaf surface with visible stomatal pores. A photograph of a portion of this nail polish film was taken at 200x magnification, and the number of stomata in each image was counted.

As comparisons were only being made between two means, control of entire experiment error was not needed, and statistical comparisons were made between means using Student's t-test with an alpha of 0.05.

Results

Rates of pod abscission under drought stress

For the first count of reproductive pods, which took place two weeks after flowering, Fuji had approximately three times the number of pods per plant than Zorro (Figure 7). In the second count of pods, which took place four weeks after flowering, the number of pods per plant for both genotypes was statistically the same. Fuji's number of pods decreased sharply between the first and second counts as the majority of pods abscised from their plants. Zorro also had a significant decrease in the number of pods per plant from the first count to the second count, but the magnitude of Zorro's decrease was smaller than Fuji's decrease. The number of trifoliate leaves was unvarying between varieties and between the two count periods.

Chlorophyll fluorescence of young stressed bean leaves

The parameter Fv/Fm did not vary by either genotype or water treatment (Figure 8A). The moderate drought stress to which these plants were exposed (the leaves of the drought stress treatment wilted the day after measurements) had no impact on Fv/Fm. Φ_{PSII} was more responsive to drought stress and had more variation among genotypes. Drought stress significantly lowered the Φ_{PSII} of all genotypes (Figure 8B). For the well-watered treatment, no

Figure 7 - Bean pod abscission
A graph of the average number of bean pods per plant for two different bean genotypes: the drought susceptible Fuji and the moderately drought tolerant Zorro. The plants were grown under consistent drought stress. Pod counts were taken two weeks after flowering and four weeks after flowering. Error bars represent standard error. Means that do not share letters are significantly different from each other (alpha= 0.05).

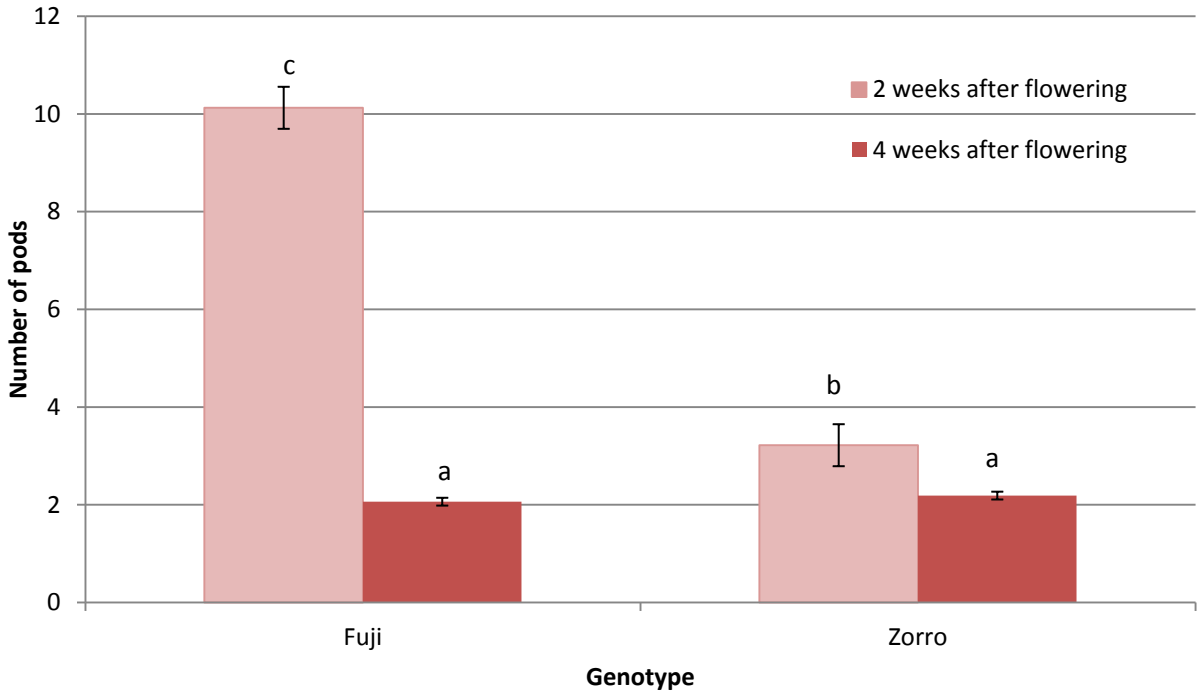


Figure 8 - Drought and chlorophyll fluorescence
(A) The average maximum photosystem II efficiency of dark adapted leaves (Fv/Fm) and (B) the average photosystem II efficiency of light exposed leaves (Φ_{PSII}) of four different common bean genotypes exposed to either well-watered or drought stress conditions. (C) The final dry weight of the aboveground biomass of the four bean genotypes after one week of exposure to either well-watered or drought stress conditions. Error bars represent standard error. Means that do not share letters are significantly different from each other (alpha= 0.05).

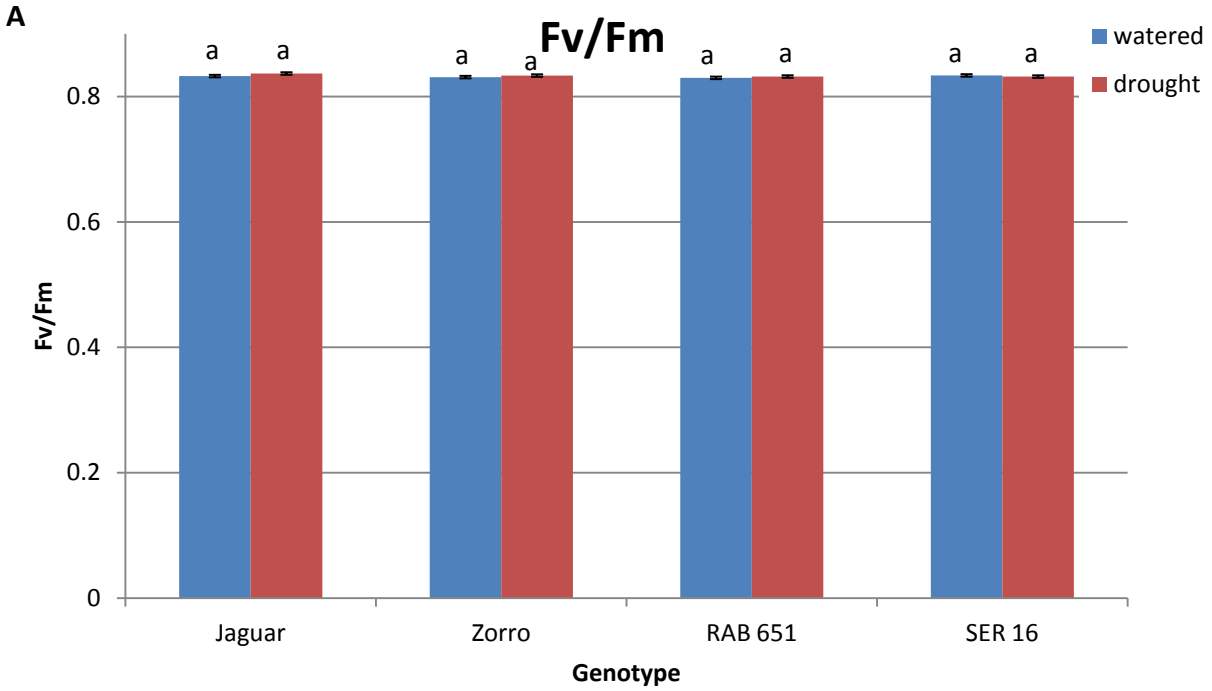
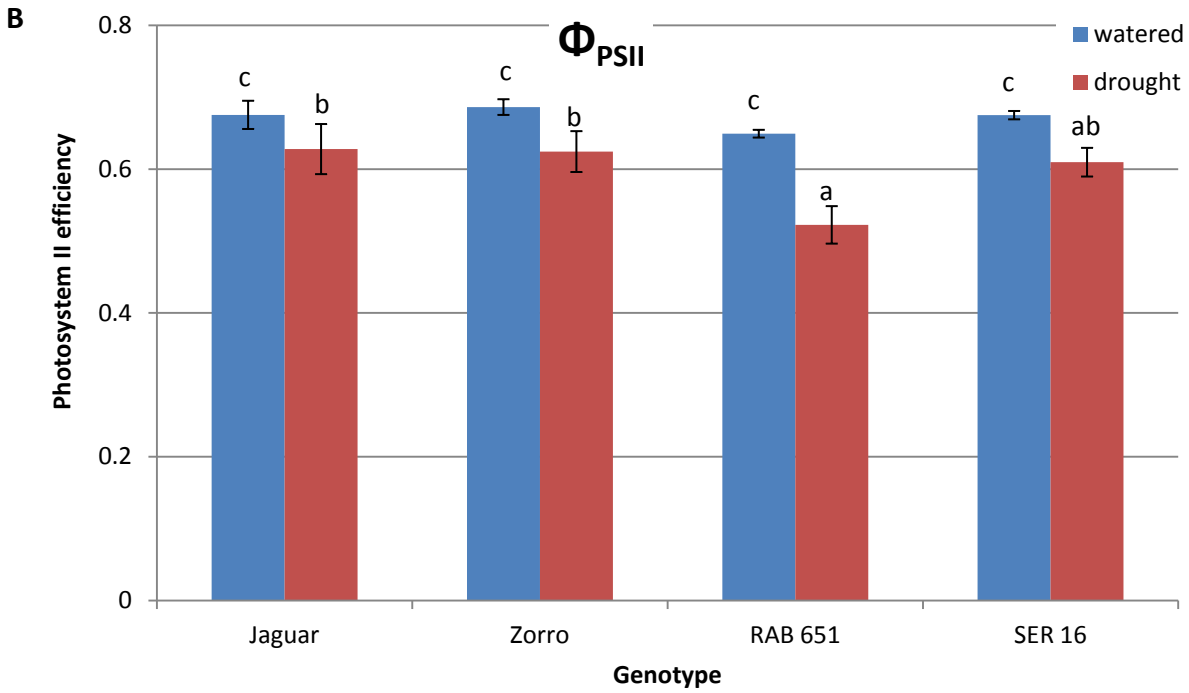
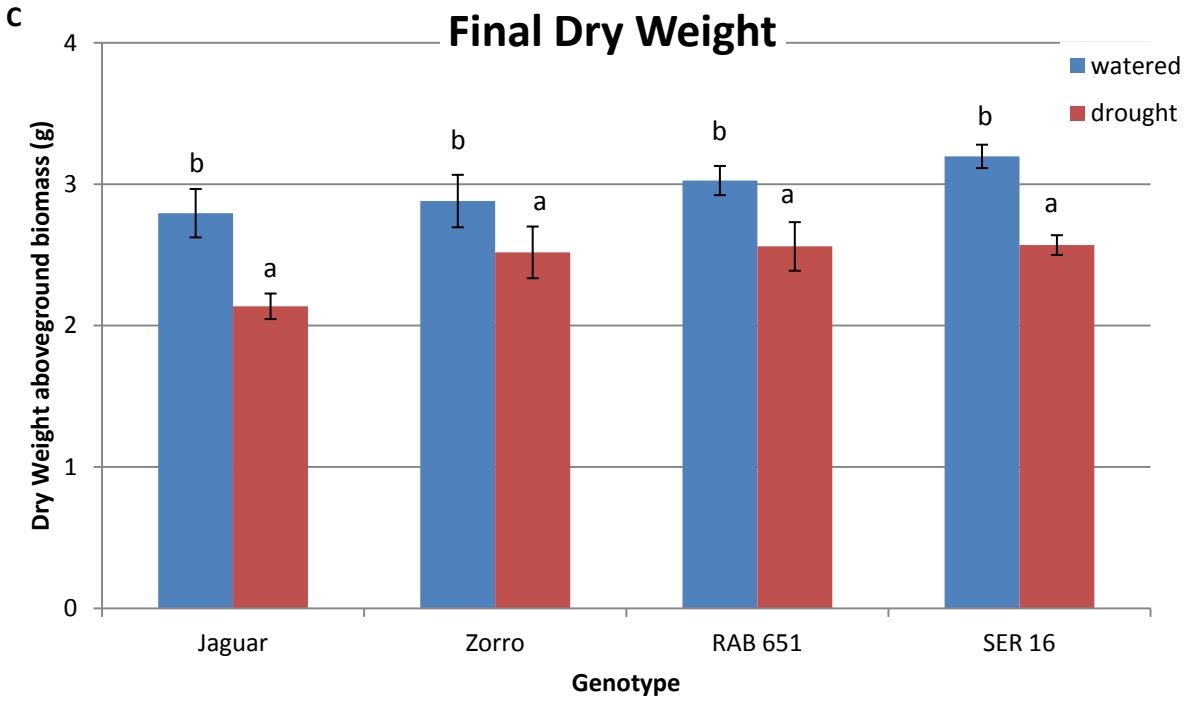


Figure 8 (cont'd)





differences were found among any of the genotypes. Under drought stress, RAB-651 had a significantly lower Φ_{PSII} than Jaguar and Zorro. Drought stress also significantly reduced the final biomass of all genotypes tested (Figure 8C). Within either water treatment, no significant differences were found among any of the genotypes.

Leaf water potential of stressed plants

Leaf water potential of drought stressed bean plants varied significantly by genotype (Figure 9). Because the plants were grown in common pots, all the genotypes in a replication experienced the same soil moisture conditions throughout the experiment and when the leaf water potential measurements were taken. At similar levels of water availability, the drought tolerant tepary had an average leaf water potential of -1.34 MPa, the lowest of all the genotypes tested. While higher than tepary, the drought tolerant SER-16 still had a lower leaf water potential than either Jaguar or Zorro. Drought susceptible Jaguar and moderately tolerant Zorro had leaf water potentials that were essentially identical and were the highest of all genotypes tested.

A-C_i curves

Representative A-C_i curves for each of the four genotypes measured are shown in Figure 10. From these curves, the averages of the photosynthetic parameters V_{cmax}, J, TPU, and g_m were determined for each genotype (Table 2). No significant differences in either TPU or g_m were found among the genotypes tested. However, Zorro had a significantly higher V_{cmax} than the other genotypes tested. Furthermore, both Tepary and Zorro had a higher J than Jaguar and SER-95.

Figure 9 - Leaf water potential

The leaf water potential of four bean genotypes under moderate to severe drought stress. Error bars represent standard error. Columns with different letters are significantly different from each other (alpha=0.05).

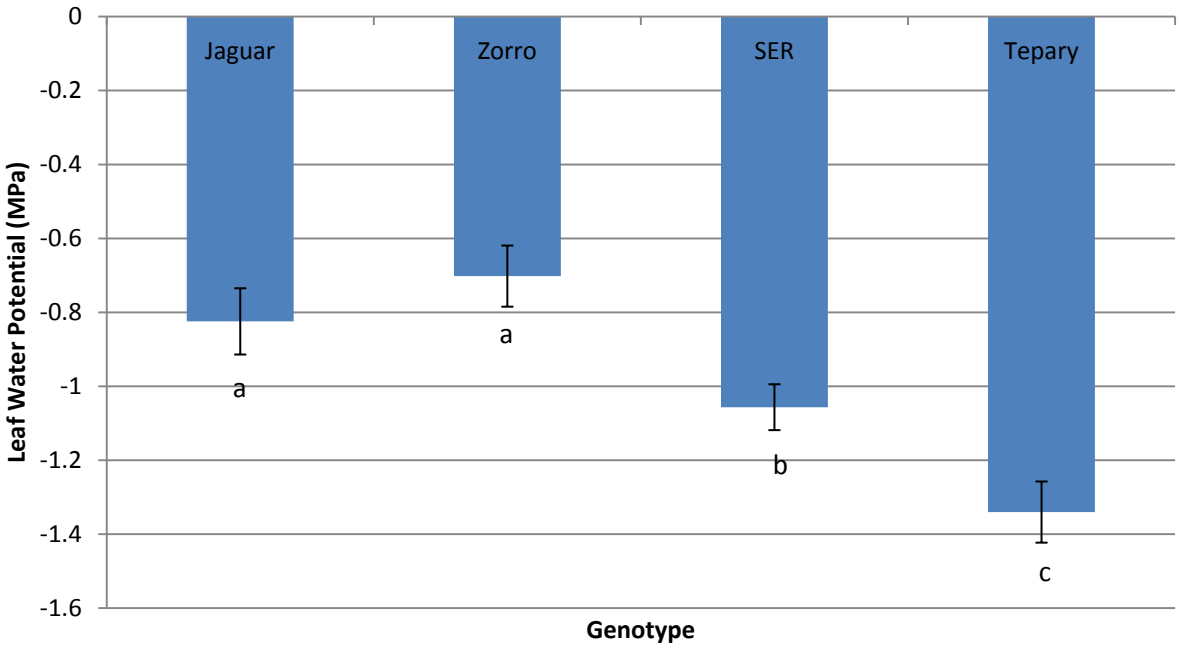


Figure 10 - A-C_i curves
Representative photosynthesis versus intercellular CO_2 concentration (A-C_i) curves of four different bean lines.

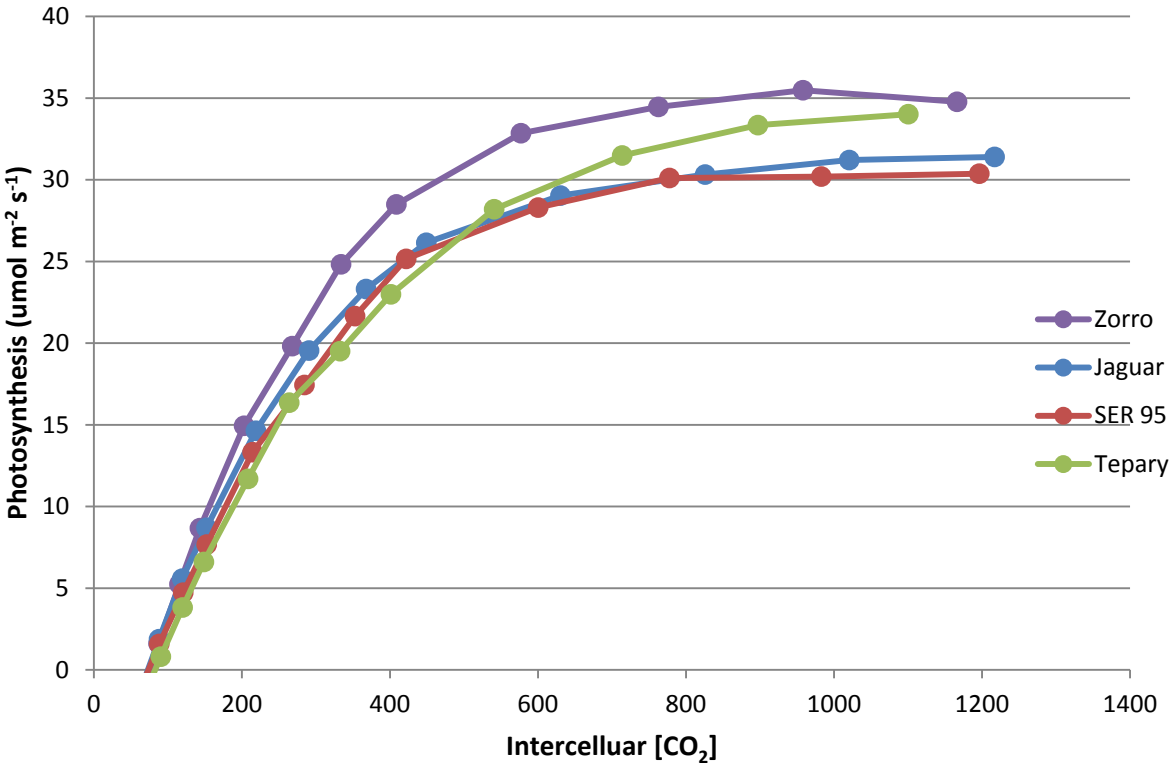


Table 2 - Photosynthetic parameters
Averages of the photosynthetic parameters of maximum carboxylation rate of rubisco (V_{cmax}), photosynthetic electron transport rate (J), triose phosphate use (TPU), and mesophyll conductance (g_m) as derived from the A-C_i curves of four different bean lines. Means within a row that share no letters are significantly different from each other (alpha = 0.05).

Bean line	Jaguar	SER-95	Tepary	Zorro
Photosynthetic parameters				
V_{cmax}	98 a	82 a	101 a	124 b
J	129 a	129 a	139 b	145 b
TPU	8.9 a	9.1 a	9.6 a	9.7 a
g_m	1.89 a	2.12 a	2.21 a	1.64 a

Legend: V_{cmax} - maximum carboxylation rate of rubisco (µmol m^{-2} s^{-1}), J - photosynthetic electron transport rate (µmol m^{-2} s^{-1}), TPU - triose phosphate utilization (µmol m^{-2} s^{-1}), g_m - mesophyll conductance (µmol m^{-2} s^{-1} Pa^{-1})

Gas exchange measurements over increasing drought stress

Both genotype and water treatment affected rates of photosynthesis (Figures 11A and 12A). The well-watered treatment had stable photosynthetic rates over the course of the measurement period with no appreciable day to day differences. Within the well-watered treatment, Jaguar and Zorro had the highest rates of photosynthesis, SER-16 was intermediate, and tepary had the lowest photosynthesis under these conditions. Under drought stress conditions, the photosynthesis of all genotypes decreased as the pots dried from evaporation and transpiration. Generally, Jaguar and Zorro had the highest photosynthetic rates under drought stress, SER-16 was intermediate (Figure 11A) or equal to tepary (Figure 12A), and tepary had the lowest photosynthesis; the relative performance of genotypes was similar to that under well-watered conditions. However, under the most extreme stress, all genotypes had similarly low photosynthetic rates. Jaguar and Zorro also recovered their photosynthesis to a greater extent than SER-16 or tepary after the pots were rewatered (Figure 11A). Photosynthesis was unaffected at pot water contents of 70% of maximum capacity (Figure 12A), but by a pot capacity of 30%, photosynthesis was moderately impacted, and by 13% it was severely impacted. During the course of the experiment, the well-watered treatment's average pot capacity stayed within the range of 75-85%.

Stomatal conductance had the same general trends as photosynthesis, and as stress increased in the drought treatment, conductance decreased as well (Figures 11B and 12B). Again, Jaguar and Zorro had the highest stomatal conductance, followed by SER-16, and finally tepary with the lowest conductance.

Figure 11 - Gas exchange over days
The measurement of gas exchange parameters on four bean genotypes subjected to two treatments: a well-watered control (dotted line with closed circles) and a progressively increasing drought stress treatment (solid line with closed triangles). A) The photosynthetic rate of these genotypes over the course of nine days. The drought stress treatment was rewatered once on day six to examine rates of recovery. B) The stomatal conductance of the same plants from (A) C) The leaf water potential of the latest fully expanded trifoliate from the well-watered and drought stress treatments at day 9 from the plants measured in (A-B). Means that share no assigned letters are significantly different from each other (alpha=0.05). All error bars represent standard error.

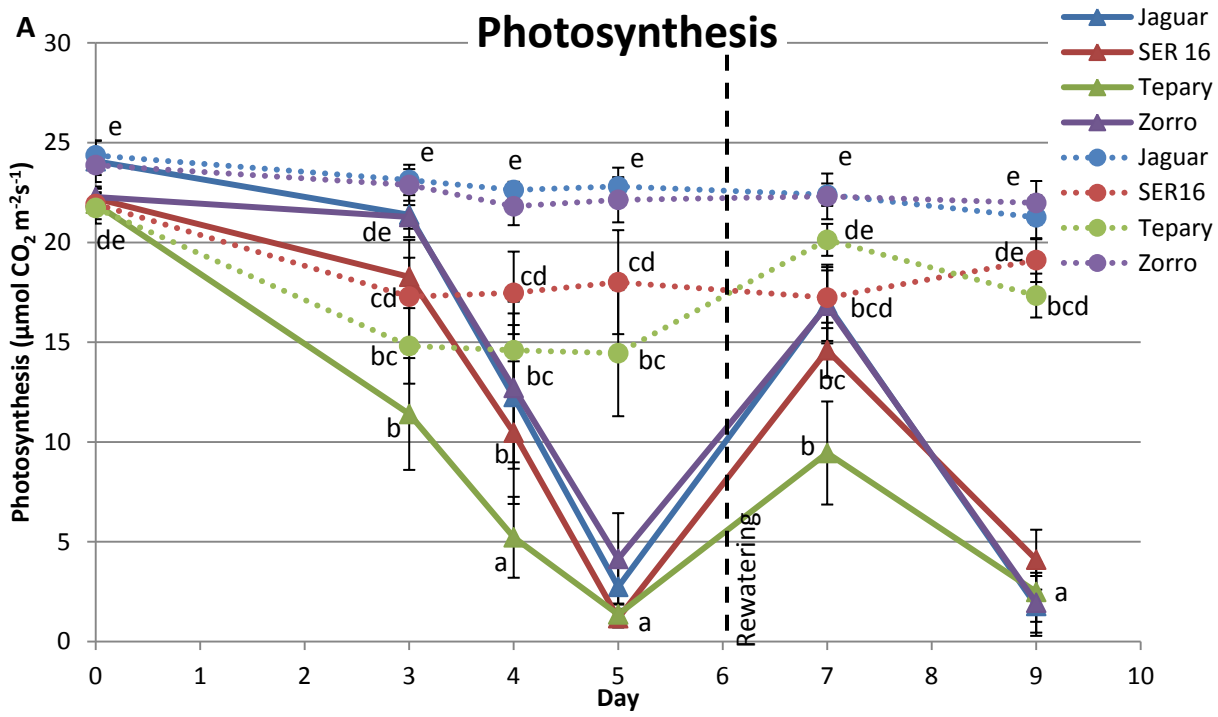
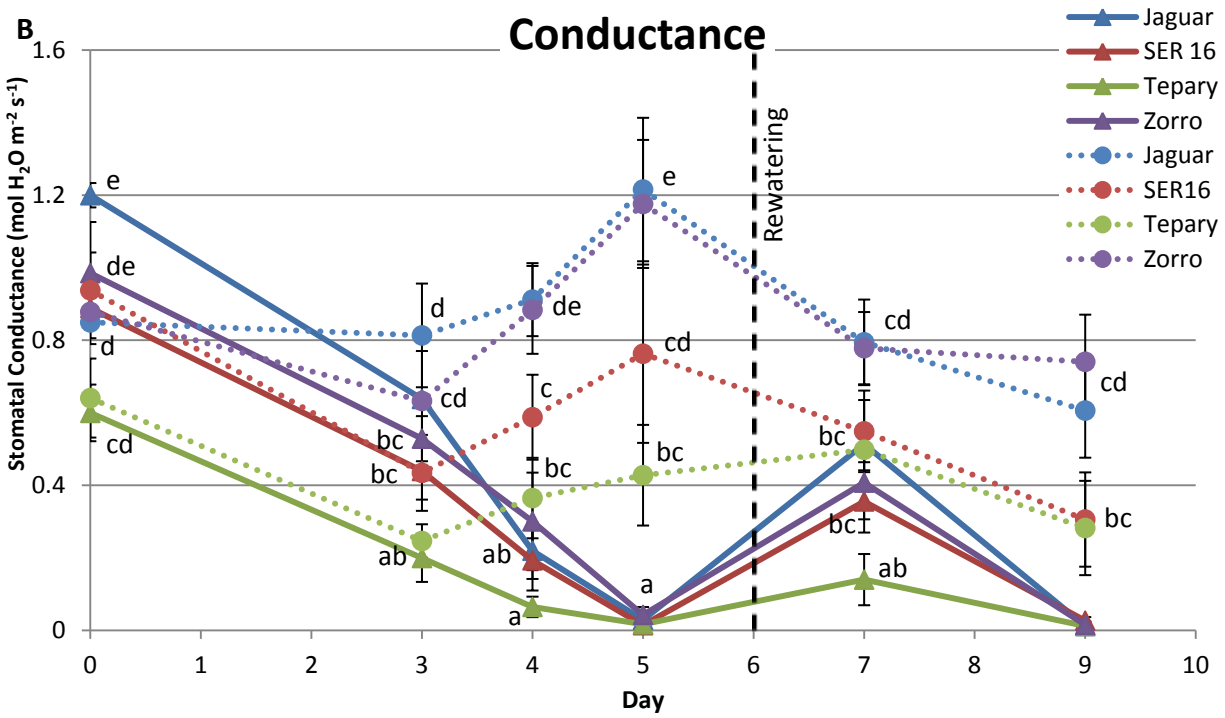


Figure 11 (cont'd)



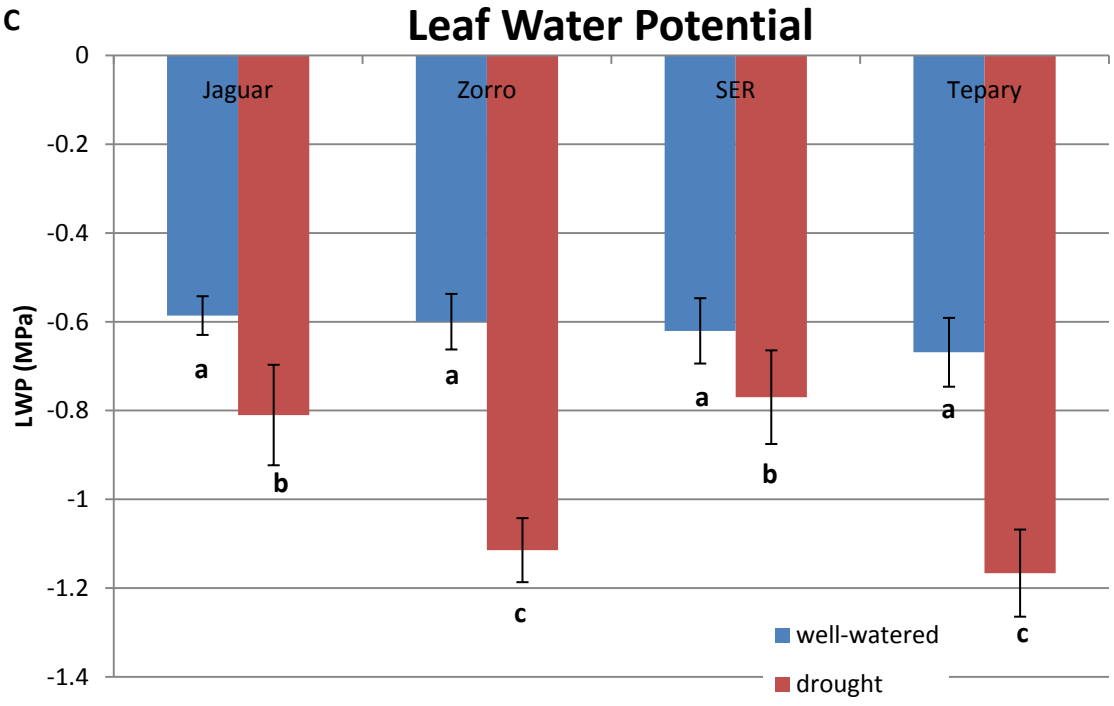
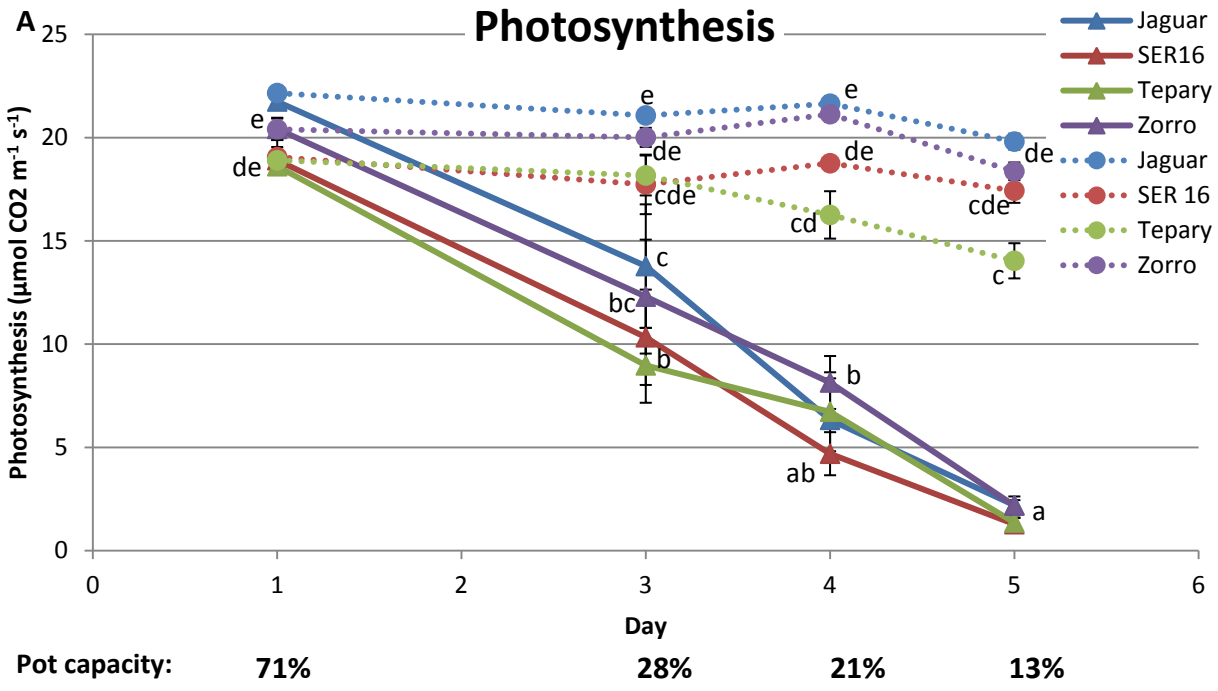


Figure 12 - Gas exchange with pot weights
The measurement of gas exchange parameters on four bean genotypes subjected to two treatments: a well-watered control (dotted line with closed circles) and a progressively increasing drought stress treatment (solid line with closed triangles). A) The photosynthetic rates of these genotypes over the course of five days. The average pot water capacity of the drought stress treatment is listed below each day of measurements. B) The stomatal conductance of the same plants from (A). Means that share no assigned letters are significantly different from each other (alpha=0.05). All error bars represent standard error.



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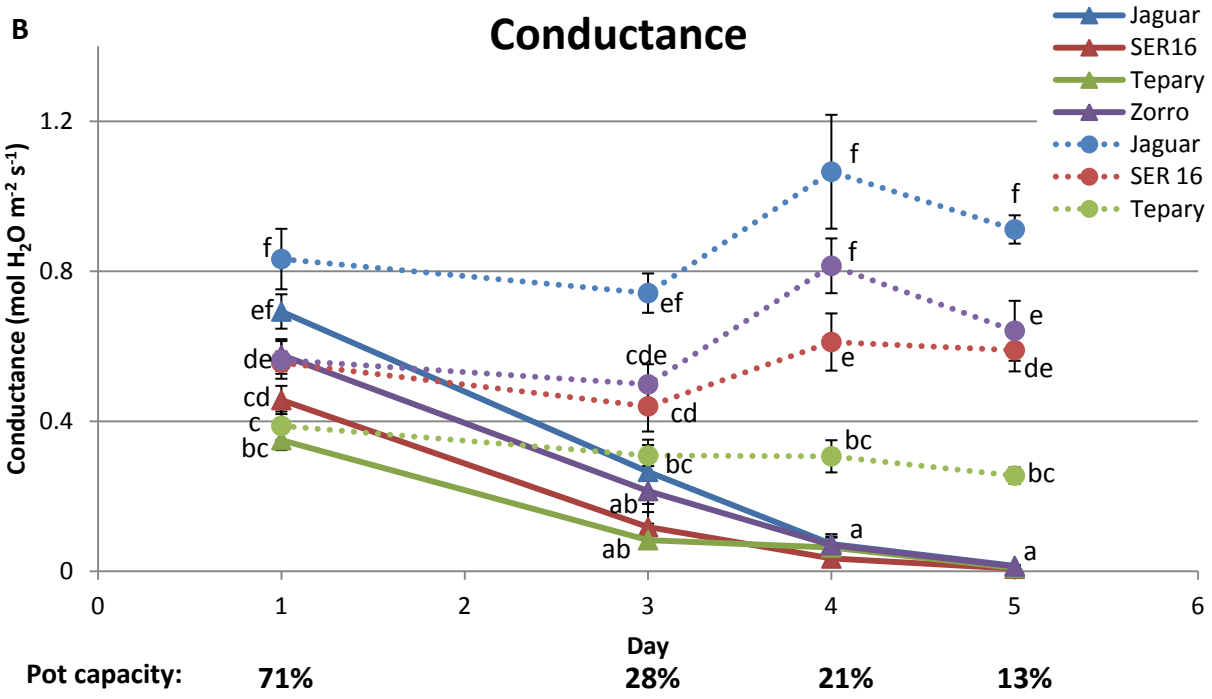


At day nine of the measurement period (Figure 11A), leaf samples from all treatments and genotypes were taken, and the leaf water potential of these samples was determined (Figure 11C). At the point of sampling, the drought treatment was experiencing severe stress. Under well-watered conditions, all genotypes had similar leaf water potentials. The drought stress treatment had lower leaf water potentials than the well-watered control, and significant differences arose among genotypes in this treatment. Jaguar and SER-16 had higher leaf water potentials than Zorro and tepary under drought stress.

Survey of tepary varieties

The ANOVA found that genotype and stress level significantly affect photosynthesis and stomatal conductance, and within certain treatments, significant differences exist within the tepary germplasm and between the tepary germplasm and Zorro, the common bean check. Under mild drought stress, differences were found among the tepary varieties for both photosynthesis and stomatal conductance (Table 3). Those varieties with higher photosynthetic rates also had commensurately higher stomatal conductance: varieties such as Tohono O'dham and San Ignacio. Additionally, the two wild tepary varieties also had higher photosynthesis and conductance than some of the domesticated varieties. However, under mild drought stress, Zorro had much higher photosynthesis and conductance than any tepary variety tested.

For severe drought stress, few differences existed for the genotypes tested, and all gas exchange rates were low. Wild tepary - Chihuahua, Tohono O'dham, and Zorro all had higher photosynthetic rates at this level of stress, but only Tohono O'dham had a higher stomatal conductance. Under recovery conditions, Zorro again had much higher photosynthesis and conductance than any tepary variety although it did not recover to levels it attained under mild

Table 3 - Tepary genotype survey
The average rate of photosynthesis (A) and stomatal conductance (g_s) for eight tepary bean genotypes and the elite common bean Zorro after exposure to mild drought stress, severe drought stress, and recovery from drought stress. Within a column, means that share no letters are significantly different from each other (alpha = 0.05).

Bean lines	*Gas exchange traits*					
	A			g_s		
	Mild	Severe	Recovery	Mild	Severe	Recovery
Zorro	19.5 a	4.3 a	12.5 a	0.585 a	0.03 ab	0.223 a
Wild tepary -Chihuah	13.6 b	2.8 bc	5.4 b	0.175 bc	0.019 b	0.058 b
San Ignacio	13 b	1.6 c	4.7 b	0.239 b	0.009 b	0.044 b
Wild tepary -Tiburón	12.2 bc	3.5 ab	2.9 b	0.186 bc	0.024 ab	0.025 b
Tohono O'dham	12 bc	4.8 a	4.1 b	0.171 bc	0.048 a	0.042 b
Big Fields Brown	11.2 bcd	2.4 bc	4.2 b	0.141 cd	0.014 b	0.049 b
Black Tepary	9.8 cd	1.8 c	4.8 b	0.137 cd	0.011 b	0.05 b
Sacaton White	8.4 de	1.5 c	5.9 b	0.099 cd	0.01 b	0.058 b
Tucson Brown	6.3 e	1.8 c	3.1 b	0.057 d	0.011 b	0.024 b
LSD	3.1	1.3	3.1	0.095	0.026	0.041
Mean	11.8	2.7	5.3	0.199	0.02	0.064

Legend - A - photosynthetic rate (µmol CO_2 m^{-2} s^{-1}); g_s - stomatal conductance (mol H_2O m^{-2} s^{-1}); LSD - Least Significant Difference, using an alpha of 0.05

stress. All the tepary varieties had slight increases in their gas exchange under recovery compared to severe stress, but no tepary variety had higher rates than any other during recovery, and all their rates were approximately a third to a half of what they were under mild stress.

Gas exchange of grafted beans

Photosynthesis decreased in response to increasing drought stress for all graft types (Figure 13A). Overall, a Jaguar shoot on a Jaguar root had the highest photosynthetic rates during the drought period while Jaguar/tepary had the second highest rates. Tepary/Jaguar had the lowest photosynthetic rates, and tepary/tepary had rates intermediate between Jaguar/tepary and tepary/Jaguar. By the most severe drought stress five days after watering, most graft types had similarly low photosynthetic rates. Stomatal conductance had more pronounced differences. Jaguar/Jaguar and Jaguar/tepary had similarly high conductances, especially under mild to moderate drought, while tepary/Jaguar and tepary/tepary had substantially lower conductances in comparison (Figure 13B). Tepary/Jaguar and tepary/tepary also maintained higher intrinsic water use efficiencies than the other two graft types throughout the experiment (Figure 13C). Five days after watering, gas exchange in all leaves was too low and variable to derive any meaning from water use efficiency.

Leaf density and stomatal density

Average leaf density was not significantly different between Jaguar and tepary. Both genotypes had fresh leaf densities that were approximately 0.018 g cm^{-2}. Average stomatal density was not significantly different between Jaguar and tepary. Their stomatal density was approximately 230-238 stomata per mm^2.

Figure 13 - Gas exchange in grafted plants
Reciprocal and self-grafts of drought susceptible Jaguar and drought tolerant tepary exposed to progressively increasing drought stress. Water was withheld after day 0, and the pots dried down over the subsequent days. (A) Average photosynthesis measurements for each graft type during each day of the drought stress period. (B) Average stomatal conductance during this period. (C) Average water use efficiency during this period. Outside marker color indicates shoot genotype, inside marker color represents root genotype; blue represents Jaguar and green represents tepary. Solid lines indicate self-grafts, and dashed lines indicate interspecific grafts. Error bars represent standard error. Means that do not share letters are significantly different from each other (alpha = 0.05).

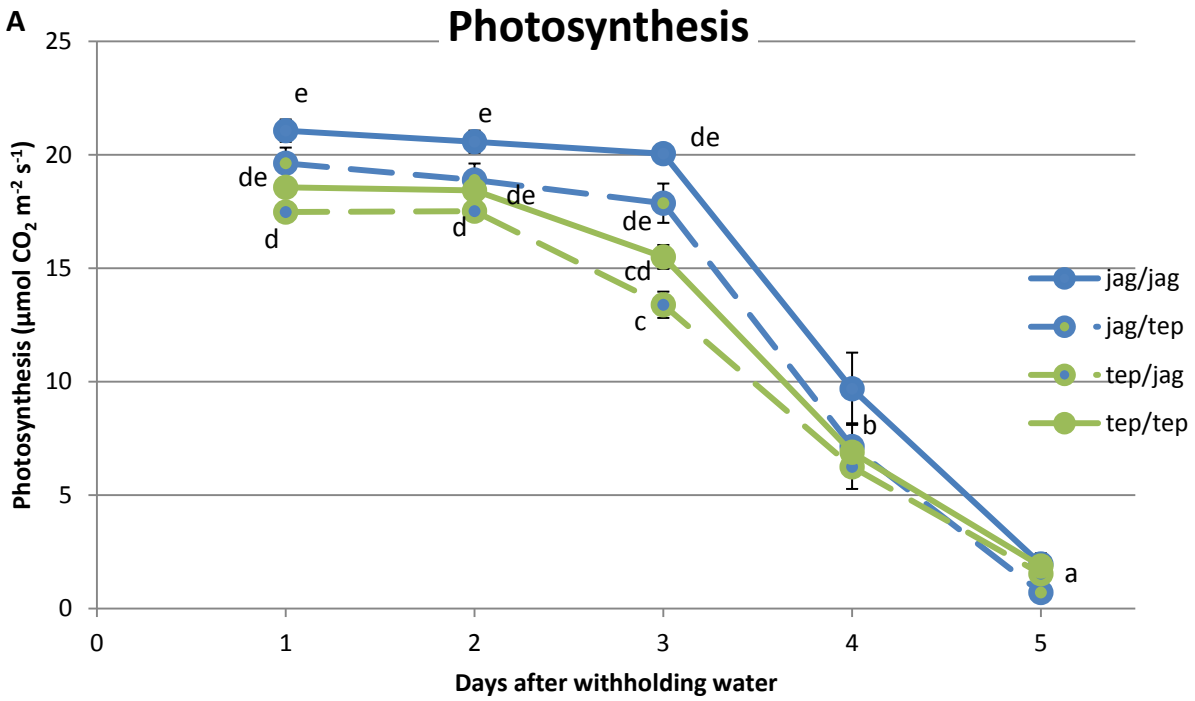
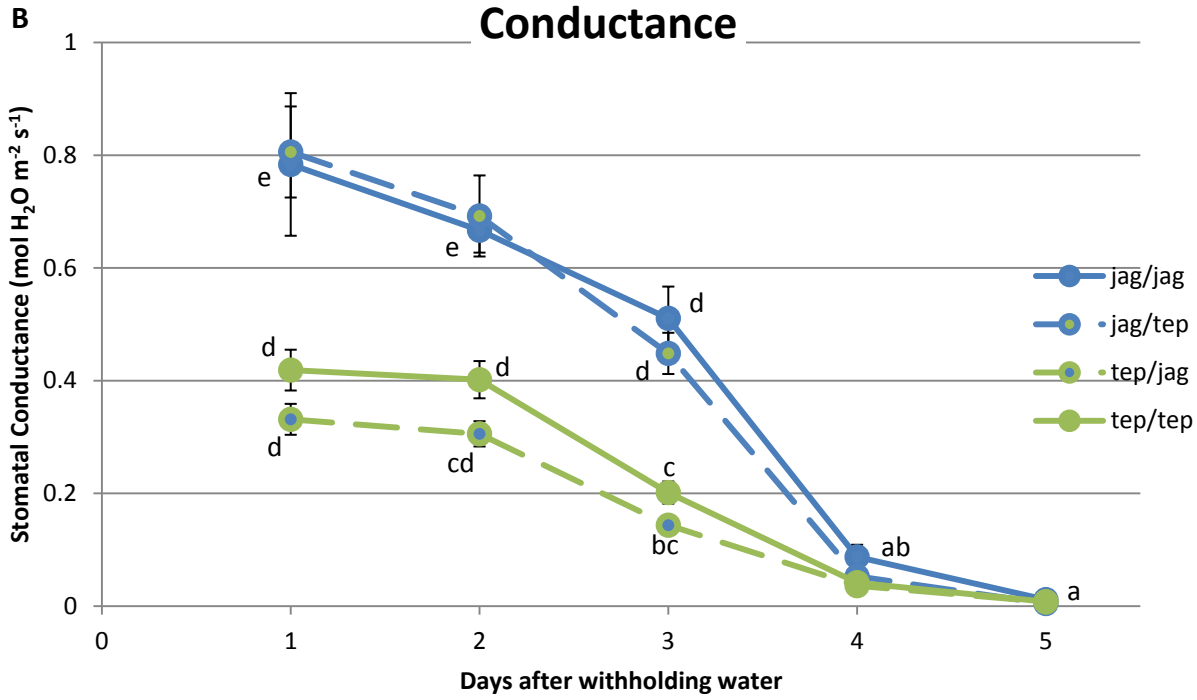
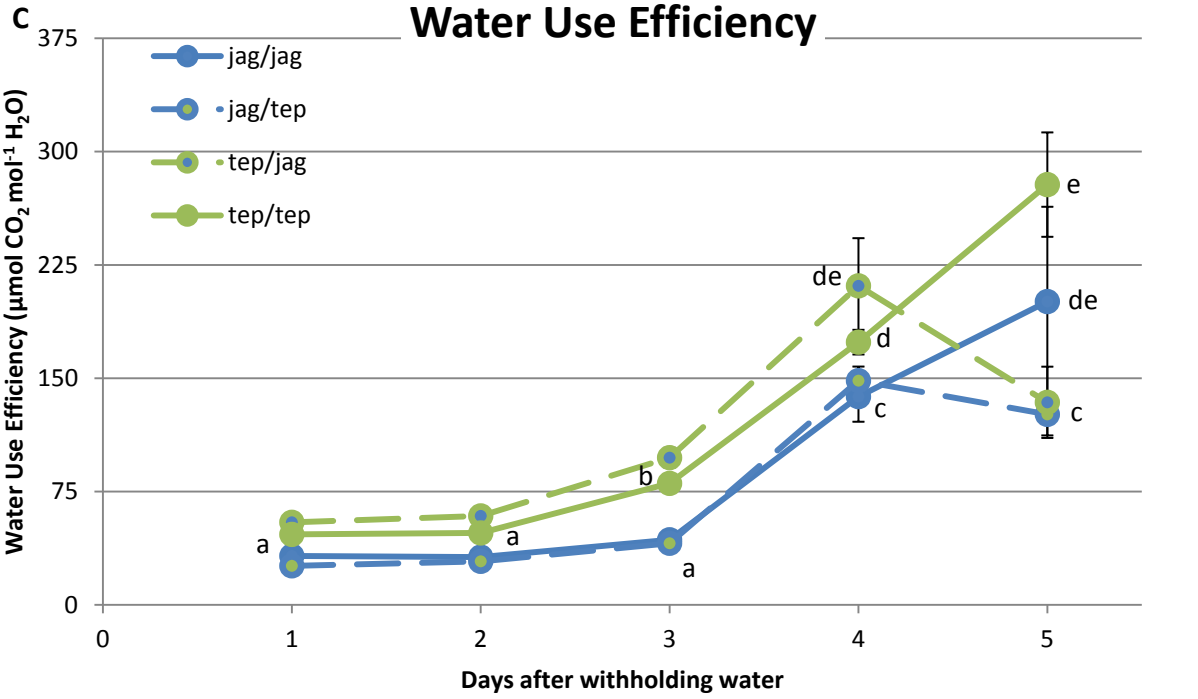


Figure 13 (cont'd)





Discussion

Effects of drought on pod abscission, water potential, and chlorophyll fluorescence

The common bean genotype Fuji had a high rate of pod abscission under drought stress, especially when compared to the Zorro. Fuji's high abscission rate explains in part its poor agronomic performance under drought stress conditions. The effects of drought on plants in their seed-filling stage is well characterized although most of the research has been done on cereal crops (Boyer and Westgate, 2004; Bahieldin et al., 2005; Tolk et al., 2013). The seed-filling stage is especially crucial: in maize, plants exposed to only three days of drought stress had the same decrease in kernel number as plants with a prolonged exposure to drought (Westgate and Boyer, 1986). The abortion of developing seed embryos under drought stress is not caused by the lack of water to embryo but by the lack of photosynthate available to the embryo (Gengenbach, 1977; Boyle et al., 1991). However, low water availability reduces rates of photosynthesis, and low photosynthetic rates reduce the amount of photosynthate available to the embryos unless reserves from other tissues, such as the stem, are remobilized (Yang and Zhang, 2006). Supply of photosynthate is thus connected closely to reproductive development, and the higher concentrations of soluble sugars that we see in some drought tolerant genotypes exposed to drought stress (Chapter 2) likely contribute to their reproductive success and yield. One of the few studies in common beans found that a drought tolerant variety and a drought susceptible variety had a proportionally similar reduction in pod and seed number between control and drought treatments, but the drought tolerant variety had higher seed yield in all cases (Boutraa and Sanders, 2001). However, the authors did not state whether the reduced yield was because of reduced pod formation or increased pod abortion. A similar experiment found no difference in

pod number under control conditions but that the drought susceptible bean genotype had a much higher rate of pod abscission than the drought tolerant genotype (Lizana et al., 2006).

In the current study, although Zorro and Fuji ended having the same number of pods per plant, the moderately drought tolerant Zorro formed fewer pods initially and aborted fewer pods after two weeks while the drought susceptible Fuji formed a large number of pods initially and aborted many of them over the course of the two weeks. Differences in plant architecture likely played a large role in differences seen between Fuji and Zorro. Fuji has a Type I determinate bush architecture while Zorro has a Type II indeterminate bush architecture. Under well-watered conditions, bean plants with Type I determinate bush architecture abscise a greater number of pods during the pod-filling stage of development than bean plants with other architectures (Izquierdo and Hosfield, 1983). Ultimately, Fuji expended more photosynthate on pods that were ultimately unproductive. Zorro's greater ability to regulate development allowed it allowed it to waste less photosynthate under the photosynthesis-limited condition of drought.

Even moderate to severe levels of drought stress do not perturb Fv/Fm (Figure 8A). The stresses that photosystem II faced during the day, as seen in a decrease in Φ_{PSII} (Figure 8B), were not sufficient to permanently damage it, as Fv/Fm was able to recover after the dark period. Granted, the plants had an entire ten hours to dark adapt and recover from any stresses during the day. A shorter period of dark adaptation might have had a Fv/Fm still affected by transient or slow-relaxing photoinhibition, but this information is already more accurately conveyed by changes in Φ_{PSII} (Baker, 2008). In both common bean, tepary bean, and cowpea, Fv/Fm did not vary between control and even severe drought stress (Castonguay and Markhart, 1991; Souza et al., 2004) while drought stress significantly impacted Φ_{PSII} and related parameters like photochemical quenching and electron transport rate (Souza et al., 2004; Wentworth et al.,

2006). Both heat and drought stress affected Φ_{PSII} at mild to moderate levels, but Fv/Fm did not shift until long exposure to extreme conditions of stress (Havaux, 1992). A wheat screening study did find small variations in Fv/Fm under stress, but it used detached leaves, its period of dark adaption was only 30 minutes, and even then no differences were found until the leaves were exposed to a combination of high light and high temperature directly preceding the dark adaption (Sharma et al., 2012).

The decrease in Φ_{PSII} under drought stress mirrors the decrease in accumulated biomass under drought stress (Figure 8C). However, while RAB-651 had a significantly lower Φ_{PSII} than Jaguar and Zorro under drought stress, no significant differences in biomass were found among genotypes in the drought stressed treatment. Averaging both the well-watered and drought stress treatments, Jaguar's biomass is significantly lower than that of RAB-651 or SER-16. Φ_{PSII} is much more responsive to drought stress and is a better parameter for screening for drought response than Fv/Fm.

The drought tolerant tepary and SER-16 had lower leaf water potentials under drought stress than the more drought susceptible Jaguar and Zorro (Figure 9). The physical flow and retention of water in plant tissues has been well studied with regards to stress. A water stressed drought tolerant soybean genotype had lower leaf hydraulic conductivity than other genotypes (Sinclair et al., 2008), which could lead to lower leaf water potentials. Tying leaf water potential to the abscisic acid work of Chapter 2, abscisic acid decreased xylem conductance (Chaves et al., 2003) but increased root hydraulic conductivity (Thompson et al., 2007). Thus, stress and abscisic acid causes more water to flow into the plant through the roots but decreases how quickly it moves through the plant and how quickly it escapes through the leaves. More drought tolerant genotypes like tepary and SER-16 thus conserved water by creating and tolerating lower

leaf water potentials. The more susceptible genotypes Jaguar and Zorro had higher leaf water potentials, so the water in their leaves was more likely to transpire out of the plant from the leaf and its stomates. The increase in soluble sugars could also play a role in decreasing leaf water potential (Chapter 2). However, while tepary had the highest concentration of soluble sugars (Chapter 2) and the lowest leaf water potential (Figure 9), SER-16, despite having a lower concentration of soluble sugars, still had a lower leaf water potential than Zorro. Thus, other factors beyond the measured solute accumulations play a role in controlling leaf water potential; however, the ability to create and maintain lower leaf water potentials aids plants in surviving and yielding under drought stress.

Photosynthesis and drought

The photosynthetic parameters V_{cmax} and J vary significantly among bean genotypes while TPU and g_m do not (Table 2). Although no differences were found in the g_m for these genotypes as calculated from A-C_i curves, using direct measurement methods instead of estimation would likely yield more accurate and precise values (Sharkey, 2015); methods such as carbon isotope discrimination (Evans et al., 1986) or combined A-C_i and chlorophyll fluorescence (Flexas et al., 2002) could reveal unseen differences. That the A-C_i curves were performed on non-stressed plants in this experiment could also contribute to a lack of differences. The g_m of grapevines decreases in response to drought stress (Flexas et al., 2002), and both interspecific and intraspecific differences in the response of g_m to increased temperature have been found (Pimentel et al., 2013; von Caemmerer and Evans, 2014). It seems likely that drought-induced differences in g_m would also exist among bean germplasm as well. That no differences in TPU were found among bean genotypes is unsurprising: TPU is rarely the limiting

rate under natural conditions (Sharkey et al., 2007), so no adaptive advantage would be gained from a higher TPU.

However, V_{cmax} and J are often limiting factors under conditions that plants face in the field, and it is for these parameters that genotypic differences were found. V_{cmax} is the limiting factor under low C_i while J is the limiting factor under high C_i (Long and Bernacchi, 2003). Because drought stress induces stomatal closure and lowers C_i, Zorro's higher V_{cmax} could contribute to its productivity under moderate drought stress; however, this would be a drought tolerance mechanism unique to Zorro and not shared by either SER-95 or Tepary. Additionally, Zorro's higher V_{cmax} would give it a greater amount of photosynthate, especially under drought stress because the effect would be greatest at low C_i. This improved supply of photosynthate could in turn be transported to developing reproductive tissues to prevent their abortion and abscission, thus accounting for Zorro's lower rate of pod abscission (Figure 7). The higher J of both Tepary and Zorro could contribute to their higher general productivity, but it is unlikely to contribute to their productivity under drought stress as it would not be a limiting factor at the low C_i of drought stress. However, Zorro yields more than other genotypes within its market class, including Jaguar (Kelly et al., 2009*a*), and this productivity could be due to its higher J that operates under favorable conditions. Likewise, Tepary behaves in some regards like a desert ephemeral (Nabhan and Felger, 1978), so its high J would make it more productive after the seasonal rains of the Sonoran Desert when water, heat, and light are all plentiful for a brief period.

Bean genotype and water treatment predictably affected photosynthetic rates and stomatal conductance (Figures 11 and 12). The four genotypes had different performances under well-watered conditions, and although drought stress caused both photosynthesis and stomatal

conductance to decrease in all genotypes, their relative ordinal performance was unchanged; i.e., Jaguar and Zorro had the highest rates of photosynthesis and stomatal conductance, SER-16 intermediate, and tepary the lowest. In all treatments and genotypes, photosynthetic rates followed the same trends as stomatal conductance. This was an expected result, as drought stress induces stomatal closure, which in turn limits the flow of CO_2 to the sites of carbon fixation, thus lowering photosynthetic rates (Chaves and Oliveira, 2004). Interestingly, while drought susceptible Jaguar and moderately tolerant Zorro had higher stomatal conductances, it was the more drought tolerant SER-16 and tepary that had lower stomatal conductances under both drought and well-watered conditions (Figure 11B). Because stomata are the primary site of water loss from plant tissues, the regulation of their opening and closing also impacts a plant's water use and water content (Medrano et al., 2002; Flexas et al., 2004; Daszkowska-Golec and Szarejko, 2013). While causing a slight reduction in photosynthesis under well-watered conditions, SER-16 and tepary's lower stomatal conductances may allow them to survive and yield under water limited conditions.

In soybean, leaf hydraulic conductance was unchanged by exposure to drought stress while stomatal conductance and leaf water potential decreased (Locke and Ort, 2014), again emphasizing the importance of stomatal control to drought response. Other studies on drought stress and gas exchange in common beans also found a strong connection between photosynthetic rates and stomatal conductance, but they either used few genotypes (Rosales et al., 2012) or uncharacterized genotypes (Ramalho et al., 2014), preventing any extrapolation of a genotype's level of drought tolerance and its rates of photosynthesis and conductance. The current study also adds a greater temporal resolution to onset and progression of drought stress in *Phaseolus*. In *Eucalyptus* spp. originating from different regions, those species adapted to hot,

dry environments had higher water use efficiencies than those from cooler, humid environments, even when all species were grown in the same common garden with mild to moderate drought stress (Héroult et al., 2013). The drought tolerant SER-16 and tepary also had higher water use efficiencies, achieved by their lower stomatal conductances (Figure 11B). Getting closer agronomically and genetically, comparisons between a cultivated soybean variety (*Glycine max* (L.) Merr.) and a stress tolerant wild relative (*Glycine soja* Sieb. and Zucc.) showed that *G. soja* reduced its transpiration to a greater extent than cultivated soybean as their soil gradually dried (Seversike et al., 2014). *G. max* and *G. soja.* are analogous to common bean and tepary bean, and in both cases, it was the stress tolerant related species (*G. soja* and tepary) that control to a greater extent the water flowing out of their stomata.

While tepary's lower stomatal conductance under drought stress had been examined previously (Markhart, 1985; Castonguay and Markhart, 1992), here the results also showed that drought tolerant common bean genotypes also have a similar response to drought stress. Previous studies reported no differences in the leaf water potential of common bean and tepary bean under drought stress (Castonguay and Markhart, 1991, 1992), but those studies imposed stress via quick acting methods of applying osmotica and dry air streams to detached leaves. In the present experiments, even when drought stress was imposed by withholding water and allowing potting soil to dry gradually, different results were obtained: the prolonged and more severe stress caused Zorro to have a significantly lower leaf water potential (Figure 11C) while a shorter and more moderate stress caused SER-16 to have a lower leaf water potential (Figure 9). Given the variation that arose when drought stress was imposed by withholding water, it is unsurprising that using different methods of imposing drought stress would produce different results.

The approximate pot water capacities at which stomatal conductance and photosynthesis were affected were also examined (Figure 12). Special care should be taken in translating this information to field conditions; the height of the water column in the pots was much shorter than a typical water column in the field, so the suction force is much weaker for the pots, causing them to have a higher absolute water content for a given relative capacity (Passioura, 2006). For that reason, the term "pot capacity" is used instead of the more familiar "field capacity".

Variation and grafting of tepary

While variation was found within the tepary germplasm tested, the variation between the tepary germplasm and a model common bean genotype, represented by Zorro, was still greater (Table 3). Given the variation within tepary, future breeders may want to carefully consider which accessions of the tepary genepool to include in future introgressions into common bean germplasm so that they continue to build on the advances of previous introgressions (Mejía-Jiménez et al., 1994; Muñoz et al., 2004, page 200; Rao et al., 2013; CGIAR, 2015). Such considerations would also be important in efforts to improve domesticated tepary germplasm, as even this basic survey indicated that sufficient variation on which improvements could be built exists within the tepary genepool. When considering tepary bean as an ideotype for breeding drought tolerant common bean lines (Blum, 2011; McClean et al., 2011; Beebe, 2012), not only did all the tepary lines have lower stomatal conductances compared to Zorro at the onset of stress, but they also had lower conductances well into the recovery phase (Table 3). This slow recovery of stomatal conductance would be especially advantageous in production areas that experience terminal drought stress. In these areas, after the onset of water stress, any subsequent precipitation is likely to be low in volume and transient. By not immediately recovering stomatal

conductance, increasingly scarce water resources are conserved further into the end of the season. However, it is important to note that Zorro's quicker recovery of stomatal conductance would be beneficial in production areas that experience intermittent drought (Beebe et al., 2012). These stomatal behaviors also match the stronger drought escape mechanisms of tepary (Nabhan and Felger, 1978) in comparison to common bean. The drought escape of tepary would give it a yield advantage under terminal drought conditions but a yield disadvantage under intermittent drought conditions compared to a common bean with weaker drought escape mechanisms.

In previous studies involving tepary bean, usually only one tepary accession was used for comparisons to common bean (Markhart, 1985; Castonguay and Markhart, 1991, 1992; Udomprasert et al., 1995; Butare et al., 2011) although exceptions exist (Rainey and Griffiths, 2005*b*; Rao et al., 2013). Although considerations of space and time will restrict the use of multiple tepary accessions in experiments, even as they have in the present research, this survey of tepary landraces and wild accessions shows that enough variation exists that caution must be used when extrapolating results from one genotype to the entire species. Further physiological characterization of tepary germplasm should be done to complement the ecological survey of the species (Nabhan and Felger, 1978).

Grafting two disparate genotypes onto each other revealed the broad effects of root and shoot tissue on a plant's response to drought. Among other factors, tissue specific signaling contributed to the interactions between root and shoot that were seen for photosynthesis. Shoot tissues produce abscisic acid and transport it to the roots, and root ABA content is dependent on shoot identity (Chapter 2). Likewise, root tissues send drought stress signals, some of which are still uncharacterized, to shoots and leaves (Chaves et al., 2003). Additionally, the plant hormone cytokinin is produced in both the shoot and root tissue (Frébort et al., 2011) and induces cell

division and stomatal opening, acting as an antagonist to abscisic acid (Zwack and Rashotte, 2015). The production and reception of some of these signals likely varies between Jaguar and tepary. Because of these differences in signaling, even though the graft type Jaguar shoot on tepary root was intermediate between Jaguar/Jaguar and tepary/tepary in terms of photosynthesis, the tepary/Jaguar was not intermediate between the two self-grafts but instead had a lower photosynthesis than tepary/tepary.

The effects of graft type on stomatal conductance were much more apparent. Shoot genotype completely determined stomatal conductance independent of root genotype (Figure 13B). Any graft type that had tepary for its shoot tissue had a lower stomatal conductance under both well-watered and drought conditions. Thus, because of the lower stomatal conductances, water use efficiency was also primarily determined by shoot genotype (Figure 13C). Under conditions of drought, a plant avoids water stress by increasing water uptake through the roots and decreasing water loss through the leaves by closing stomata (Chaves et al., 2003). But even in the root limited environment these plants were grown in, which would prevent increased water uptake by root exploration of the soil, stomatal control still operated as an important drought avoidance mechanism for tepary. A previous study involving reciprocal grafts of common bean and tepary bean and their water relations under drought stress found that leaf water potential was determined by root genotype while the root:shoot ratio was determined by the additive effects of root and shoot genotypes (Sanders and Markhart, 1992). That study also examined stomatal conductance, but its results were inconclusive. In the current study, it was found that because of tepary shoot's lower stomatal conductance, it conserved water and avoided drought stress while still having a higher photosynthesis per unit of water lost than Jaguar shoots, all regardless of root identity.

Leaf traits

Neither density of leaf tissue nor stomatal density varied between Jaguar and tepary (Figures 7 and 8). Not only are tepary and Jaguar distinct in their level of drought tolerance, with tepary being very tolerant and Jaguar being susceptible, but they are also genetically distinct, the two being different species belonging to the same genus. Thus, if any drought tolerance-connected differences in leaf or stomatal density were to be found, they would be found between Jaguar and tepary. As only two genotypes were used, these results do not prove a lack of variation for leaf and stomatal density within *Phaseolus*, but they do indicate that these traits have no effect on the drought tolerance of genotypes within this genus. Other plant families do exhibit variation in stomatal density (Hetherington and Woodward, 2003). However, within the genus *Banksia*, no clear relationship exists between a species' stomatal density and the availability of water within its ecological niche (Drake et al., 2012). As tepary and Jaguar have the same stomatal density, the differences observed in their stomatal conductance (Figure 11B) are caused by differences in the regulation of stomatal opening. Leaf density varies not only by vegetation type but also by ecological conditions; species adapted to dry environments tend to have a higher leaf density (Reich et al., 1999; Wright et al., 2005). As tepary has small leaves, another adaptation often seen in desert-adapted plants, it was hypothesized that it might also have denser leaves as well, but tepary's leaves were no denser than its drought susceptible relative. Leaf density has no connection to drought tolerance within *Phaseolus*.

Conclusions

The response of beans to drought stress varied in predictable ways according a genotype's drought tolerance. Often, differences among genotypes were not apparent under well-watered conditions and only manifested when the plants experienced drought stress. Under drought stress, the more tolerant genotype shed fewer developing pods. While no differences existed under well-watered conditions, under drought stress, drought tolerant genotypes maintained lower leaf water potentials. Other drought-related traits were constitutive and observable even under well-watered conditions. Tolerant genotypes had lower photosynthetic rates and stomatal conductances under both control and stress treatments. While photosynthetic parameters such as V_{cmax} and J did not relate directly to drought tolerance, they might contribute to a plant's primary productivity, which in turn can contribute to a plant's tolerance to stress. Traits such as leaf tissue density and stomatal density did not vary and play no part in drought tolerance in *Phaseolus*. While shoot and root tissues both affected photosynthesis, shoot tissue solely affected stomatal conductance, and thus, it is the most important determinant of water loss and the water use efficiency. Significant variation exists within *P. acutifolius* germplasm in their response to drought, but the variation between *P. acutifolius* and *P. vulgaris* is likely greater. From these results, an ideotype of a drought tolerant bean can be built and used to aid breeding decisions. This drought tolerant ideotype would have low stomatal conductance under both well-watered and drought stress conditions, have a low rate of pod abscission, small leaves, and, for areas that frequently experience terminal drought, a flowering stage that could be induced by drought conditions.

Chapter 4: Heat stress screens

Introduction

Common beans (*Phaseolus vulgaris* L.) are subject to a number of stresses in their production areas. Chief among these stresses is drought, which affects a large portion of the world bean crop every year (Cavalieri et al., 2011; Beebe, 2012). Heat stress also impacts bean production, not by reducing yields to the same extent as drought but by limiting the areas where beans can be grown (Beebe et al., 2011); climate change will most likely increase the area of land unsuitable for bean production because of high temperatures. Additionally, heat stress is often coincident with drought stress in the field and can increase its severity (Beebe et al., 2013). As with many abiotic stresses, heat and drought stress induce some of the same responses in plants. Plants responses to both involve the same redox pathways (Koussevitzky et al., 2008), signaling plant hormones (Wahid et al., 2007; Cutler et al., 2010), heat-shock proteins and molecular chaperones (Wang et al., 2004), and transcription factors (Schramm et al., 2008). However, heat and drought stress also activate different transcriptional pathways involved in independent metabolic and physiological responses (Rizhsky et al., 2004; Mittler, 2006; Shulaev et al., 2008; Rasmussen et al., 2013). The extent of overlap between heat and drought tolerance in common bean has been studied little, and it remains a question whether it is the differences or the commonalities in heat and drought pathways that have the greatest practical effect in plant performance.

Much of the work done on heat stress in beans focused on its detrimental effects on reproduction. High temperatures render pollen unviable and thus prevent seed set and limit yield (Gross and Kigel, 1994; Prasad et al., 2002). Greenhouse studies successfully identified genotypes with tolerant reproductive systems that could produce seed at high temperatures

(Rainey and Griffiths, 2005*a*,*b*). Using the same principles, a screen of multiple bean lines at a field site with temperatures prohibitive to the cultivation of most beans separated heat tolerant from susceptible genotypes based on their ability to set seed (CGIAR, 2015).

In both greenhouse and field studies, tepary bean (*Phaseolus acutifolius* A. Gray) genotypes or common bean genotypes with tepary in their pedigree were well-represented among the heat tolerant group (Rainey and Griffiths, 2005*b*; CGIAR, 2015). Tepary originated in the Sonoran Desert, located in present-day northwest Mexico and southwest United States, and was used as a reliable staple crop in this region for hundreds of years (Nabhan and Felger, 1978). Compared to common bean, tepary has a great tolerance for both heat and drought stress (Castonguay and Markhart, 1992; Udomprasert et al., 1995; Rao et al., 2013). Tepary thus serves as a useful check and ideotype for abiotic stress tolerance in beans (Beebe et al., 2013).

While these previous studies focused on the effects of heat on bean reproduction, the present study examined the effects of heat stress on vegetative tissues. Field tests are potentially the best measure of crop performance, but unpredictable weather patterns, such as too much rain or low temperatures, can prevent the evaluation of a desired trait. Thus, in addition to studying the heat stress physiology of beans, the present study also tested the feasibility of using high temperatures in a controlled environment to screen germplasm for heat and drought tolerance. These screens could then be used to supplement information from field evaluations and help inform breeding decisions. A selection of genotypes, some evaluated for drought tolerance in the field and others tested in previous experiments, were subjected to gradually increasing temperatures over the course of a week in a growth chamber. The genotypes were measured periodically for photosynthetic traits and stress-related damage.

Materials and methods

Fifteen genotype screen across three temperatures

Fifteen bean genotypes were used in this experiment; ten genotypes came from a drought diversity panel and were recommended based on their diversity of drought response by Dr. Carlos Urrea of University of Nebraska - Lincoln at the Scottsbluff research station and Dr. Timothy Porch of the USDA Tropical Agriculture Research Station in Mayaguez, Puerto Rico. Five other genotypes were included from previous stress experiments (the full list of genotypes is listed in Table 4). The fifteen genotypes were planted sequentially in four blocks with one week of spacing between each block and two replications of each genotype per block. Plants were first grown in 5 L black plastic pots filled with Suremix Pearlite potting media (Michigan Grower Products, Galesburg, MI, USA) in a greenhouse set to 25 °C in East Lansing, MI from December 2014 to February 2015. Pots were fertilized with 12 g of Osmocote-15-9-12 slow-release fertilizer (Bloomington Brands, LLC, Bloomington, Indiana, USA) and supplemented twice a week by watering with half-strength Hoagland solution until the pots were saturated and dripping.

One month after its planting, when the third trifoliate was fully mature, a block would be moved into a growth chamber set to 35 °C during the light period and 30 °C during the dark period; the chamber had a 14 h light and 10 h dark cycle with a light intensity of 400 µmol photons m^{-2} s^{-1}. For each block, no more than five days elapsed between when the earliest plants had mature third trifoliates and the latest plants had them, and movement was delayed until every plant had mature third trifoliates. After two days in this environment, gas exchange and

Table 4 - Bean genotypes used in screen

A list of bean genotypes used in the progressively increasing heat stress experiment along with their parentage or origin and their level of drought tolerance. Characterization of drought tolerance in the numbered lines comes from yield data taken field plots experiencing moderate drought stress (Tim Porch, personal communication).

Genotype	Origin	Drought Response
I14538	Tacana x VAX6	Tolerant
I14539	(Morales x XAN176) x (BAT 477 x B98311)	Susceptible
I14541	Black Rhino x SEN 10	Moderately tolerant
I14544	(BelMiDak RMR 10 x B01741) x (BAT 477 x L88-63)	Tolerant
I14545	Matterhorn x SER 21	Moderately tolerant
I14546	USPT-ANT x (Matterhorn x 98078-5-1-5-1)	Moderately tolerant
I14548	Merlot x (Merlot x SER-16)	Susceptible
I14549	Merlot x (98020-3-1-6-2 x Tacana)	Moderately tolerant
I14550	Merlot x (98020-3-1-6-2 x Tacana)	Moderately tolerant
I14553	Merlot x (05F-5055-1 x 98020-3-1-6-2)	Tolerant
Fuji	Hime tebo x Matterhorn (Kelly et al., 2009*b*)	Susceptible
Jaguar	B90211 x N90616 (Kelly et al., 2001)	Susceptible
SER-95	CIAT breeding program	Tolerant
Tepary (TB1)	Breeding program, USDA-ARS Mayaguez, Puerto Rico	Very tolerant
Zorro	B00103*2 x X00822 (Kelly et al., 2009*a*)	Moderately tolerant

chlorophyll fluorescence measurements were taken on the second most recently mature trifoliate leaf. Then the temperature was raised to 40/35 °C, and the acclimation time and measurements repeated, and the temperature again raised to 45/40 °C, and the acclimation time and measurements repeated. Finally, canopy temperature and visual ratings were taken on all plants. The plants were then cleared out from the growth chamber, and the entire cycle was repeated using the next block of plants.

Gas exchange measurements of photosynthesis and stomatal conductance were made with a LI-COR 6400XT (LI-COR Biosciences, Lincoln, NE, USA). Gas exchange measurements were taken under a light intensity of 1000 µmol photons m^{-2} s^{-1}, a CO_2 concentration of 400 µmol mol^{-1}, ambient relative humidity of approximately 50-70%, and ambient temperature.

Chlorophyll fluorescence measurements of photosystem II efficiency (Φ_{PSII}) and non-photochemical quenching (NPQ) were taken using the MultispeQ as part of the PhotosynQ platform (photosynq.org).

An E30bx infrared camera (FLIR Systems, Inc., Wilsonville, OR, USA) was used to measure the leaf canopy temperature at an ambient air temperature of 45 °C. The camera was positioned 1 m above each plant and pointed perpendicular to the ground for each infrared picture taken. The temperature of the entire visible canopy was averaged and used as that plant's leaf canopy temperature.

One hour after being removed from the growth chamber and placed back in the greenhouse, each plant was rated on its qualitative appearance on a 1-5 scale; examples of this scale are found in Figure 14G. A rating of '1' represented a plant that was nearly dead, '2' a plant that showed severe damage, '3' a plant that had moderate damage, '4' a plant that had little visible damage, and '5' represented a plant that was undamaged.

The data were analyzed using the statistical software program SAS version 9.4 (SAS Institute, Cary, NC, USA). The procedure PROC MIXED was used to determine the analysis of variance (ANOVA) for various factors and if they significantly impacted the dependent variable. The data for photosynthesis, stomatal conductance, Φ_{PSII}, and NPQ were treated as repeated measures with the factors of genotype and temperature; the "repeated" statement was used in the analysis. The canopy temperature and visual rating data were treated as single factor experiments with genotype being the only contributing independent variable. The predetermined alpha cutoff value was 0.05.

Select genotype screen over increasing temperature

Having tested the effects of increasing temperature on fifteen genotypes, five genotypes were selected from that group for a more in-depth study to determine on a finer temporal scale when and at what temperatures differences in genotypes appear. The five genotypes were I14538, I14541, I14553, Jaguar, and Tepary, and they were chosen to cover a range of responses to heat stress based on the previous fifteen genotype study. Six replications of each genotype were planted in a growth chamber set to 25/20 °C light/dark temperature; other environmental conditions were the same as described above in the 'Fifteen genotype screen across three temperatures' section. After one month of growing in these conditions, the third trifoliate leaf of each plant was mature. Then, the light and dark period temperatures were raised by 5 and 4 °C, respectively, every two days until the chamber reached a maximum of 45/36 °C. Measurements of gas exchange and chlorophyll fluorescence were taken as described above on the day before the first temperature increase and every day thereafter.

Destructive measurements of electrolyte leakage and thiobarbituric acid-reactive substances (TBARS) were taken from leaf samples harvested every two days to measure stress-induced damage to cellular membranes and oxidative damage, respectively. For electrolyte leakage, 17 mm diameter leaf punches were taken from the latest fully expanded leaf of each plant. The leaf punches were immediately placed in 20 mL of deionized water. The leakage of ions from the leaf punches into the surrounding water was measured as an increase in electrical conductivity using a conductivity probe (OAKTON Instruments, Vernon Hills, IL, USA). Conductivity was measured every 30-45 minutes until the readings plateaued. Samples were then frozen in a conventional -20° C freezer to fully disrupt membranes, thawed, and conductivity was measured again. Electrolyte leakage was determined by dividing a sample's plateaued conductivity by its post thaw conductivity.

TBARS measurements were made according to the protocol in Wang et al. (2009) with slight modifications. Briefly, 0.5 g of fresh leaf tissue was ground up in 5 mL of water containing 0.1% (weight/volume) trichloroacetic acid. The solution was centrifuged, and 0.3 mL of the supernatant was combined with 1.2 mL solution of 20% (w/v) trichloroacetic acid and 0.5% (w/v) thiobarbituric acid. The resulting solution was placed in a heating block set to 100 °C for 30 minutes, after which the reaction was terminated by removing the solution and placing in an ice water bath. After cooling, the solution was centrifuged, and then its absorbance at 535 nm was read using a spectrophotometer. The TBARS concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

The data was statistically analyzed as described above in the "Fifteen genotype screen across three temperatures' section.

Results

Fifteen genotype screen across three temperatures

Among the 15 bean genotypes, variation was found for all of the responses measured. The ANOVA found that both genotype and temperature have p-values below the alpha value (0.05) and thus significantly contribute to the outcome of photosynthesis, stomatal conductance, and NPQ (Table 5). Genotypic effects also significantly affected leaf canopy temperature and qualitative visual rating (Table 5). However, Φ_{PSII}'s p-value for genotype was 0.08, above the alpha value of 0.05, and thus, genotype does not meet the criteria for being considered a significant contributor to Φ_{PSII}. Temperature was, however, a significant contributor to Φ_{PSII} (Table 5).

Photosynthesis remained fairly constant at 35 °C and 40 °C (Figure 14A). Little variation existed among genotypes within a temperature or between these two temperatures within a genotype. However, at 45 °C, differences among genotypes became apparent, and photosynthesis ranged from 0-12 μmol CO_2 m^{-2} s^{-1}. Rates of photosynthesis at 45 °C were significantly lower than those at 35 or 40 °C. Stomatal conductance was significantly higher at 40 °C than it was at 35 °C. At 45 °C, stomatal conductances fell below those at 35 °C, and genotypes that had comparatively high conductances at previous temperatures did not necessarily have them at 45 °C. At this highest temperature, a wide range of stomatal conductances was again found among genotypes, and they correlated well with photosynthetic rates at this same temperature (Table 6).

Φ_{PSII} did not vary between 35 and 40 °C, and the variation among genotypes was also small at these two temperatures (Figure 14C). When the temperature was raised to 45 °C, Φ_{PSII}

Table 5 - ANOVA of heat screen experiment

Displayed below are the p-values of the ANOVA test of the effect of the independent factors of genotype, temperature, and the interaction of genotype and temperature on the parameters of photosynthesis (A), stomatal conductance (g_s), photosystem II efficiency (Φ_{PSII}), non-photochemical quenching (NPQ), leaf temperature (L_{temp}), and visual rating (vis). An alpha value of 0.05 was used as the cutoff for determining significance. The analysis was made with the PROC MIXED statement in the statistical analysis software SAS 9.4.

Indep. factor	**P-values**					
	A	**g_s**	**Φ_{PSII}**	**NPQ**	**L_{temp}**	**vis**
Genotype	<.0001	<.0001	0.0821	<.0001	0.0380	<.0001
Temperature	<.0001	<.0001	<.0001	<.0001	-	-
Gen x Temp	0.0272	0.0251	0.0784	0.0422	-	-

Table 6 - Correlations of measured parameters in heat screen experiment
This table contains the Pearson correlation coefficients for the parameters measured in the 'Fifteen genotype screen across three temperatures' experiment. Correlated with each other are photosynthesis, conductance, photosystem II efficiency, non-photochemical quenching, plant canopy temperature, and visual rating. The analysis used data from all fifteen genotypes and three temperatures, with the exception of plant canopy temperature and visual rating: because they were only measured at the end of the experiment, these two parameters were correlated with the other parameters using only the 45 °C data. The p-values for each Pearson correlation coefficient are in parenthesis.

	A	**g_s**	**Φ_{PSII}**	**NPQ**	**T_{canopy}**	**Visual**
A	1	0.76997 (<.0001)	0.4961 (<.0001)	-0.33288 (<.0001)	-0.63448 (<.0001)	0.54955 (<.0001)
g_s	0.76997 (<.0001)	1	0.34305 (<.0001)	-0.26313 (<.0001)	-0.57997 (<.0001)	0.3371 (0.0002)
Φ_{PSII}	0.4961 (<.0001)	0.34305 (<.0001)	1	-0.63364 (<.0001)	-0.40963 (<.0001)	0.65241 (<.0001)
NPQ	-0.33288 (<.0001)	-0.26313 (<.0001)	-0.63364 (<.0001)	1	0.15189 (0.1873)	-0.31965 (0.0046)
T_{canopy}	-0.63448 (<.0001)	-0.57997 (<.0001)	-0.40963 (<.0001)	0.15189 (0.1873)	1	-0.36485 (<.0001)
Visual	0.54955 (<.0001)	0.3371 (0.0002)	0.65241 (<.0001)	-0.31965 (0.0046)	-0.36485 (<.0001)	1

Legend: A - photosynthesis, g_s - stomatal conductance, Φ_{PSII} - photosystem II efficiency, NPQ - non-photochemical quenching, T_{canopy} - plant canopy temperature, visual - qualitative visual rating

Figure 14 - Heat screen with fifteen genotypes
15 bean genotypes subjected to first two days at 35 °C, then two days at 40 °C, and finally two days at 45 °C. (A) Average rates of photosynthesis for each genotype at the three different temperatures. Measurements were taken at the end of the two day period of each temperature regime, in the mid-morning. (B) Stomatal conductance of the 15 genotypes, taken simultaneously with photosynthesis. (C) Photosystem II efficiency of the 15 genotypes, taken just preceding photosynthesis measurements. (D) Non-photochemical quenching of the 15 genotypes, taken simultaneously with photosystem II efficiency. (E) The leaf canopy temperature of each genotype, taken on the last day of their exposure to 45 °C. (F) The qualitative visual rating of each genotype one hour after the end of the 45 °C period. Plants were rated on a scale of 1 to 5, with 1 being a plant that appeared mostly dead, and 5 being a plant that appeared undamaged. (G) Representative plants from each of the five visual rating scores. All error bars represent standard error. (A-C) Genotypes are organized from greatest to least based on values at 45 °C. (D) Genotypes are organized from least to greatest based on values at 45 °C. (E) Genotypes organized from least to greatest. (F) Genotypes organized from greatest to least.

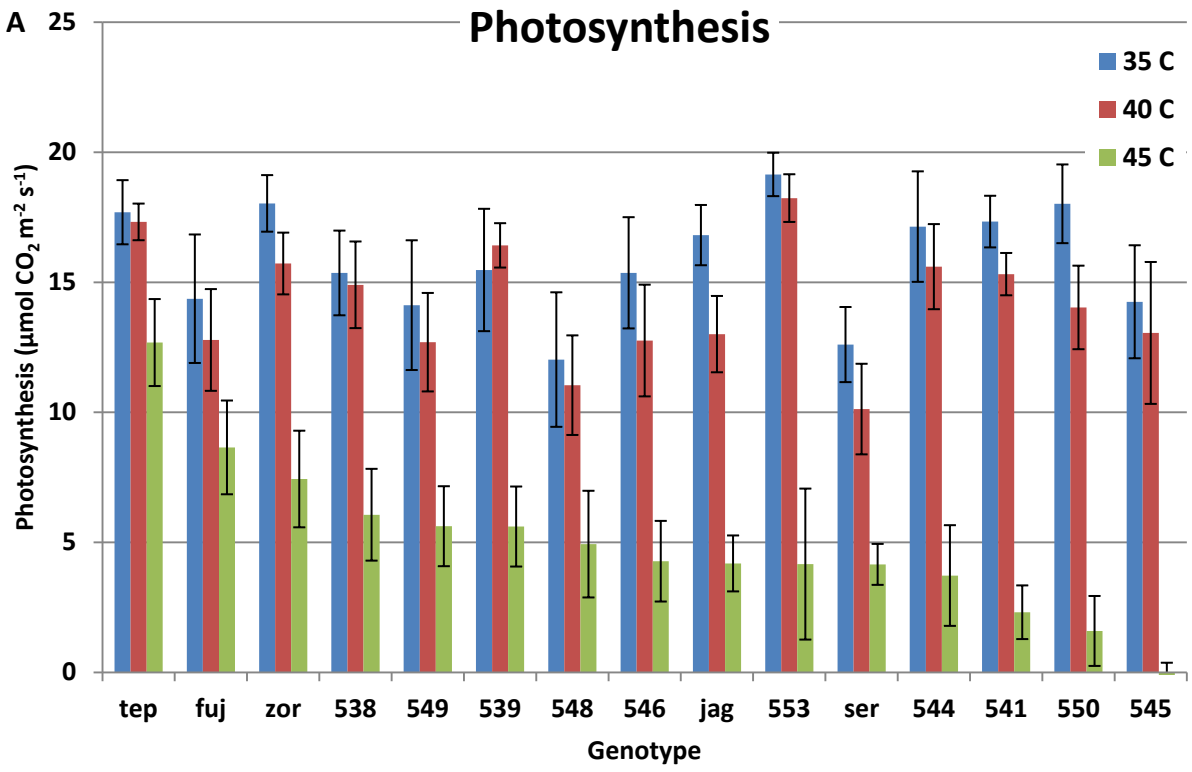
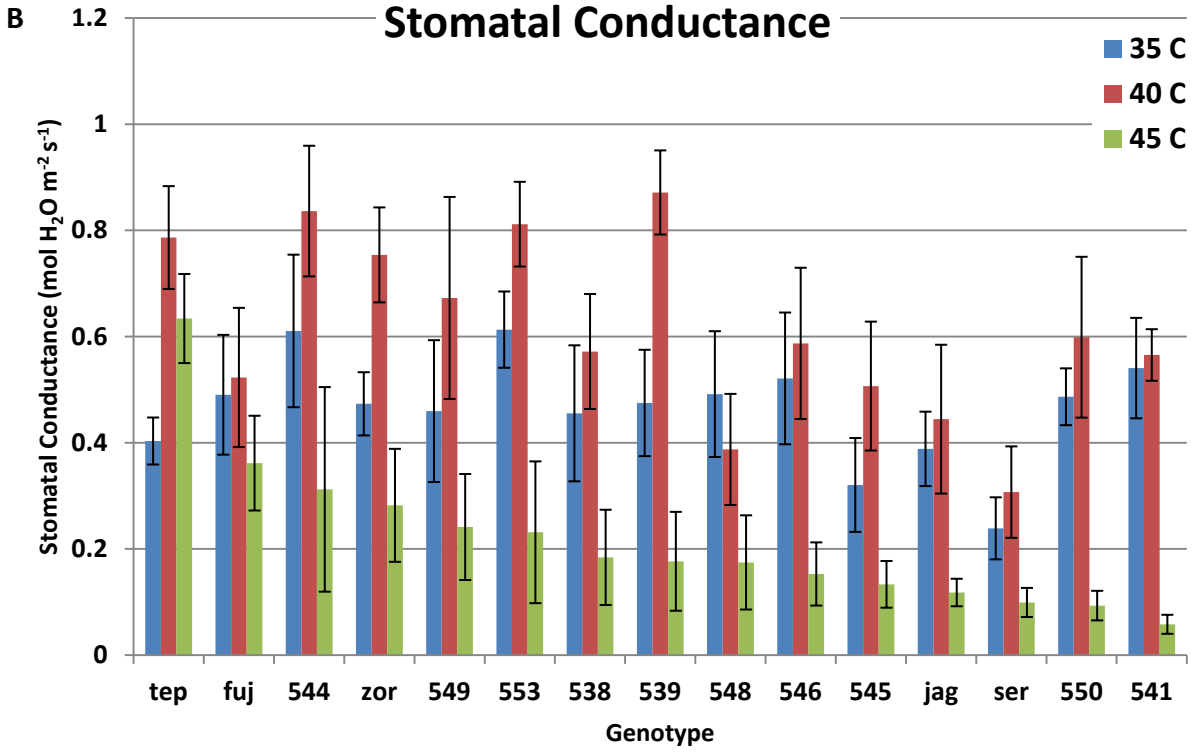
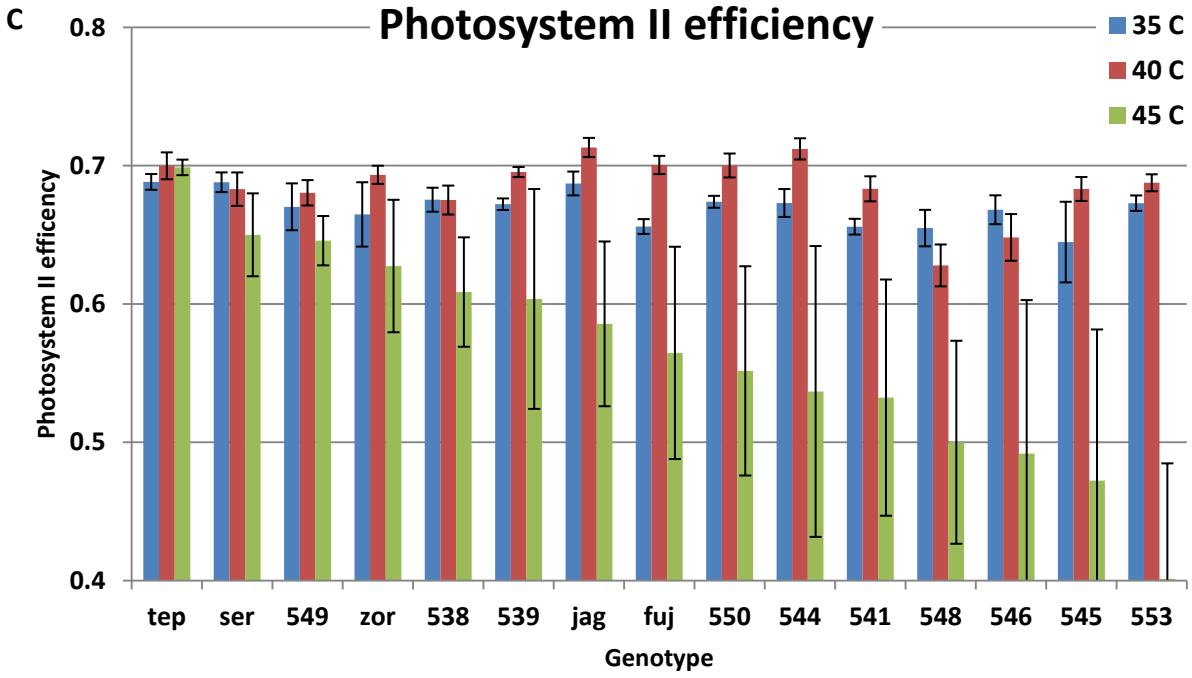


Figure 14 (cont'd)





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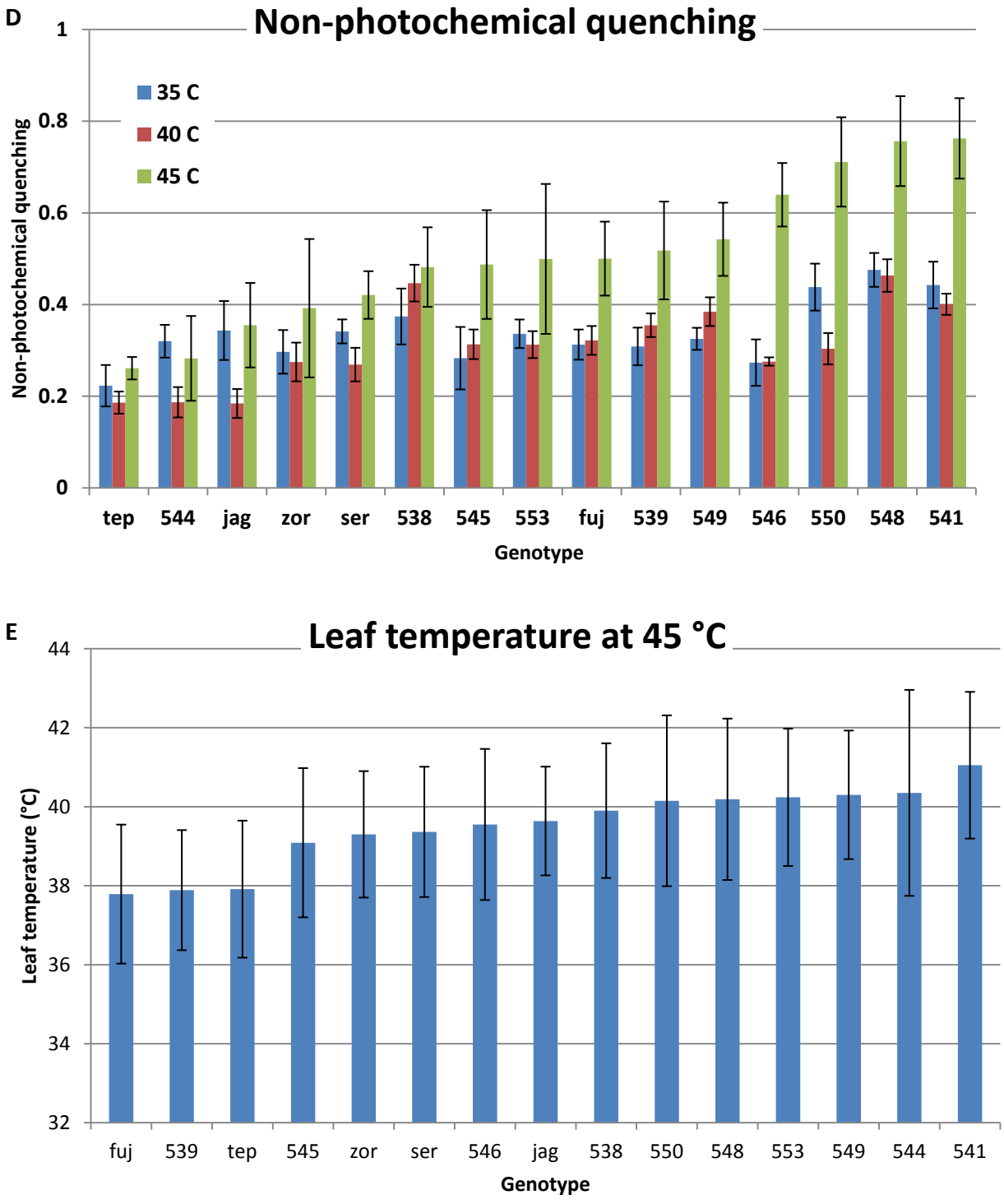

Figure 14 (cont'd)

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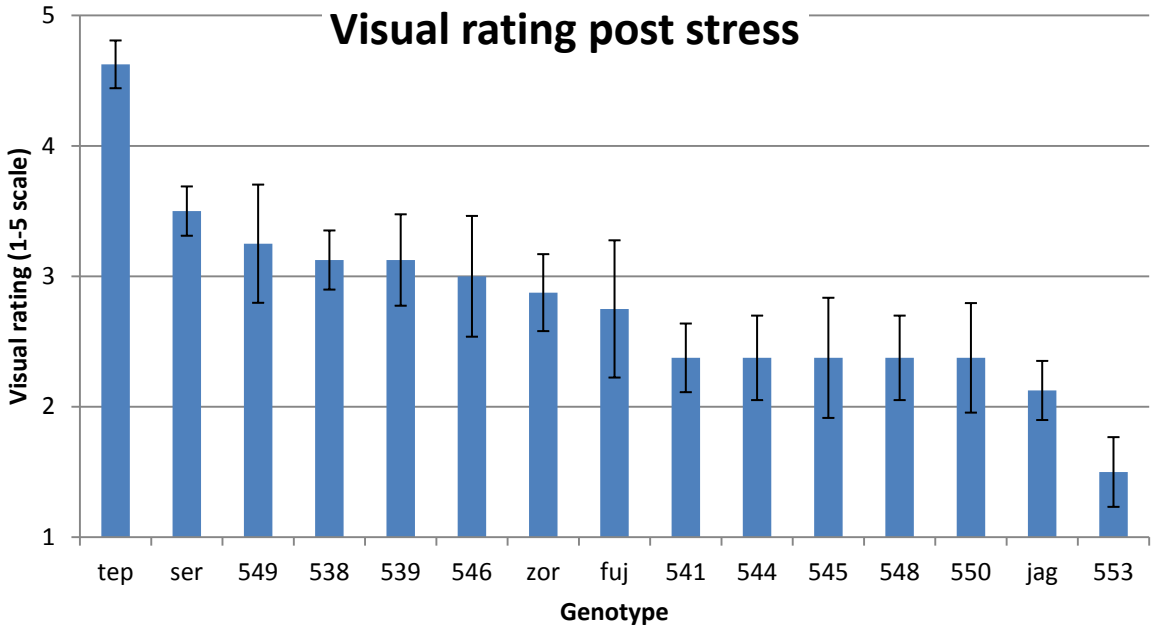


Figure 14 (cont'd)

Legend: (1) top left is a picture of genotype I14538 after exposure to 45 °C for two days, (2) top center is I14538, (3) top right is I14538, (4) bottom left is I14541, and (5) bottom center in TB1 tepary

decreased for almost every genotype. However, genotype to genotype variation also increased, and no significant differences among genotypes were found, even when the 45 °C data was analyzed on its own, separate from the other two temperatures. Like Φ_{PSII}, NPQ had few differences between temperature or genotype at 35 and 40 °C, but at 45 °C, many genotypes saw an increase in NPQ (Figure 14D). At this highest temperature, genotypes with the highest Φ_{PSII} also tended to have the lowest NPQ.

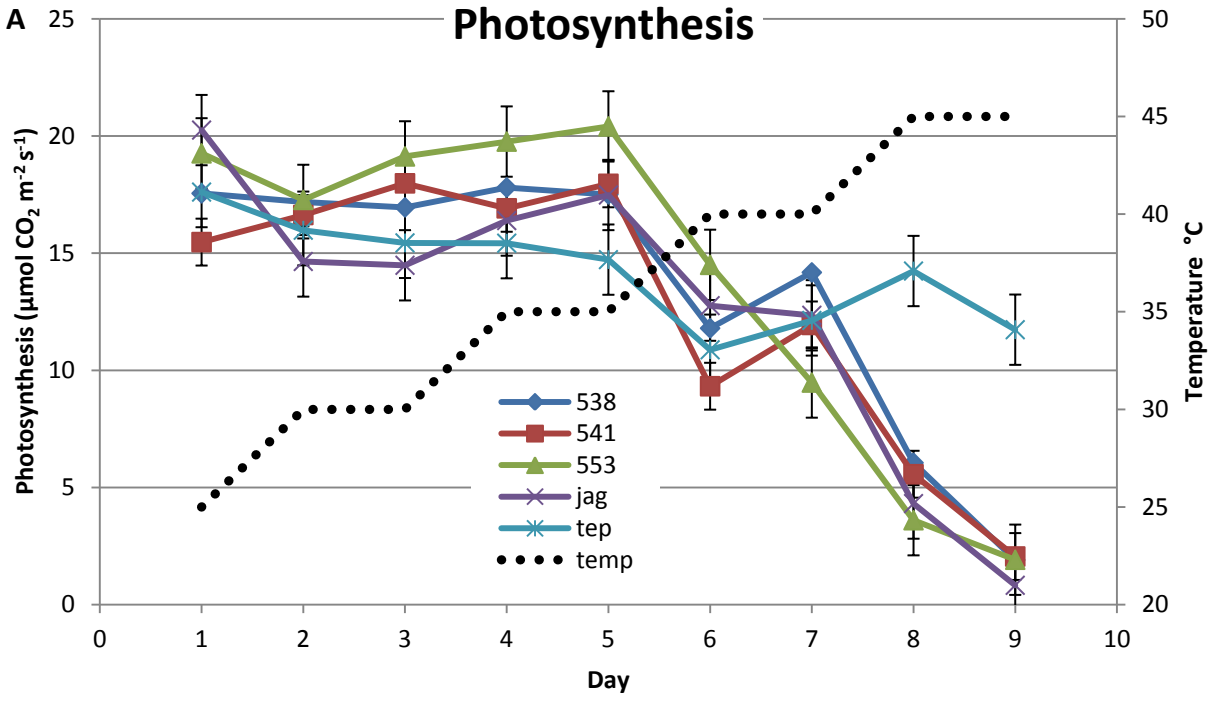
Leaf canopy temperatures at 45 °C ranged from 38 to 41 °C, but there were few significant differences among genotypes (Figure 14E). Qualitative visual ratings taken after the stress treatments showed significant differences between genotypes, and significant variation was found among the genotypes' average ratings (Figure 14F).

Select genotype screen over increasing temperature

For most measured parameters and most temperatures, the five genotypes performed similarly. Photosynthesis remained stable for all genotypes until the temperature reached 40 °C, causing a small drop in photosynthetic rates (Figure 15A). Upon reaching 45 °C, photosynthetic rates were very low, falling somewhere between 1-5 µmol CO_2 m^{-1} s^{-1}, for all genotypes except tepary. Stomatal conductance increased with increasing temperatures until temperature reached 40-45 °C; at that point, conductance decreased to low rates for all genotypes except tepary (Figure 15B).

Most genotypes had an increase in Φ_{PSII} from 25 to 30 °C, and then Φ_{PSII} was fairly stable until temperatures reached 45 °C, at which point average Φ_{PSII} values decreased greatly for all genotypes except tepary (Figure 15C). Conversely, NPQ was uniformly low for all genotypes

Figure 15 - Heat screen with five genotypes
Five bean genotypes were exposed to increasing temperatures: starting at 25 °C on day one, the temperature increased by 5 °C every two days until it reached a maximum of 45 °C at days eight and nine. (A) Photosynthetic rates of the five bean genotypes taken daily during this period. The dotted black line indicates the temperature at which each day's measurements were taken both in this graph and the following graphs in this figure. (B) The stomatal conductance of these genotypes over this period. (C) The photosystem II efficiency of these genotypes during this period. (D) The non-photochemical quenching of these genotypes during this period. (E) The TBARS content of leaf samples taken from each genotype every two days. (F) The electrolyte leakage of leaf samples taken from each genotype every two days. (G) The qualitative visual rating of each genotype at the end of the nine-day heat stress period. Plants were rated on a scale of 1-5, with 1 assigned to a plant that seemed dead and 5 being assigned to a plant that seemed undamaged. Bars that share no letters are significantly different from each other (alpha = 0.05). See Figure 14G above for example pictures of each rating. All error bars represent standard error.



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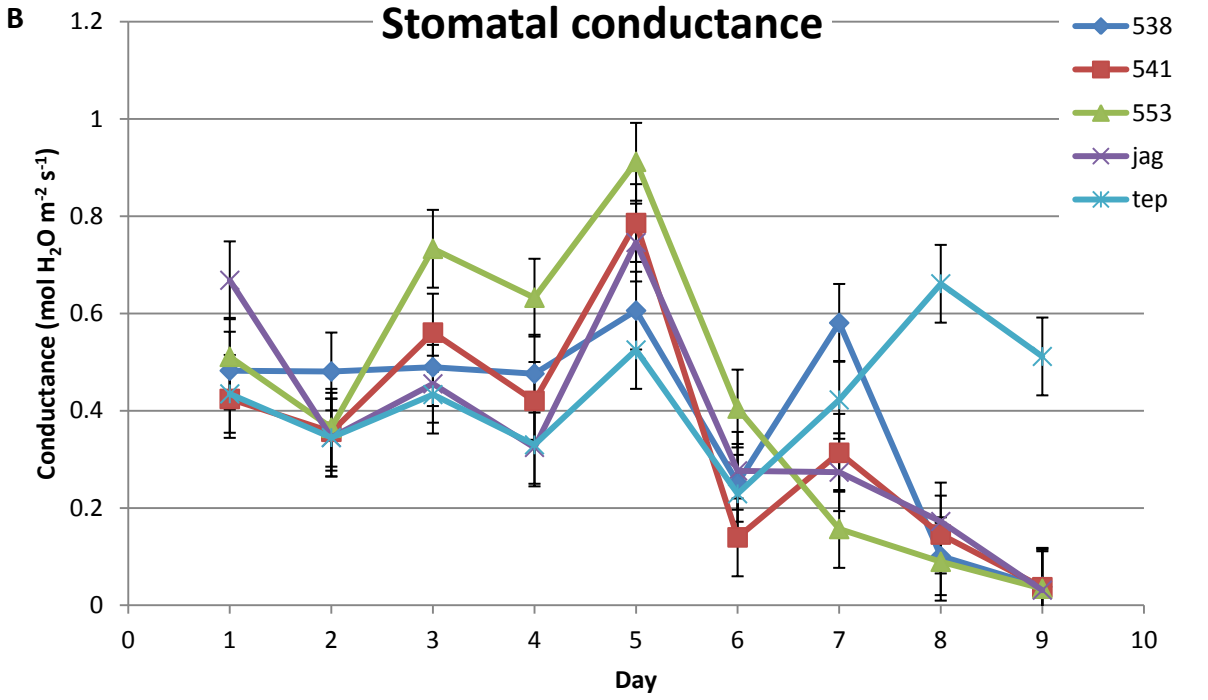


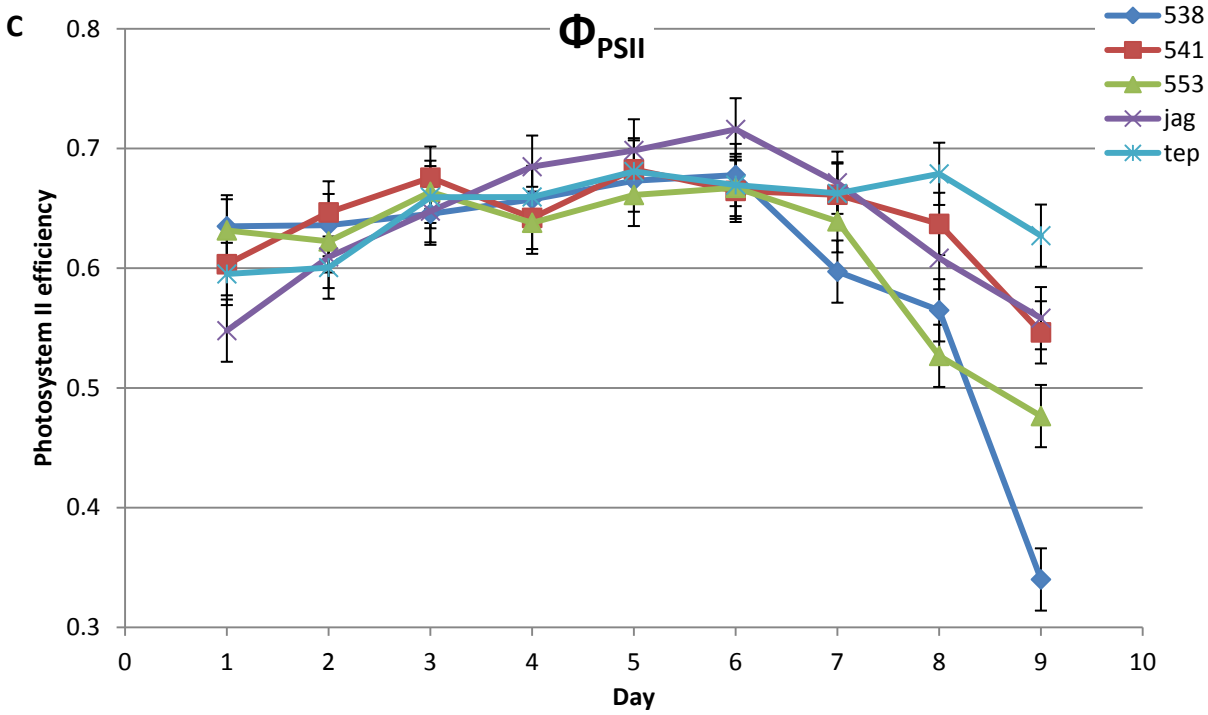
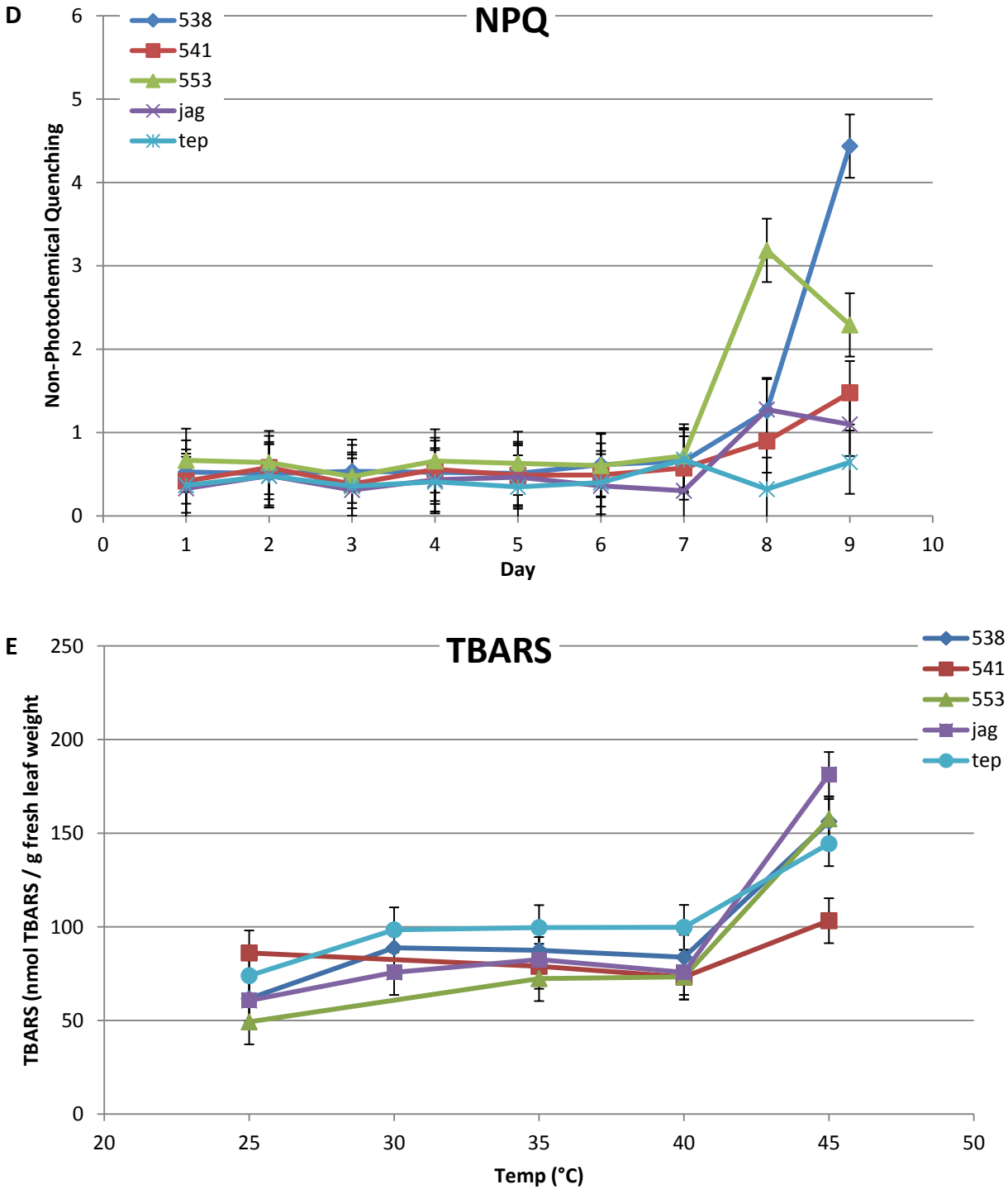


Figure 15 (cont'd)



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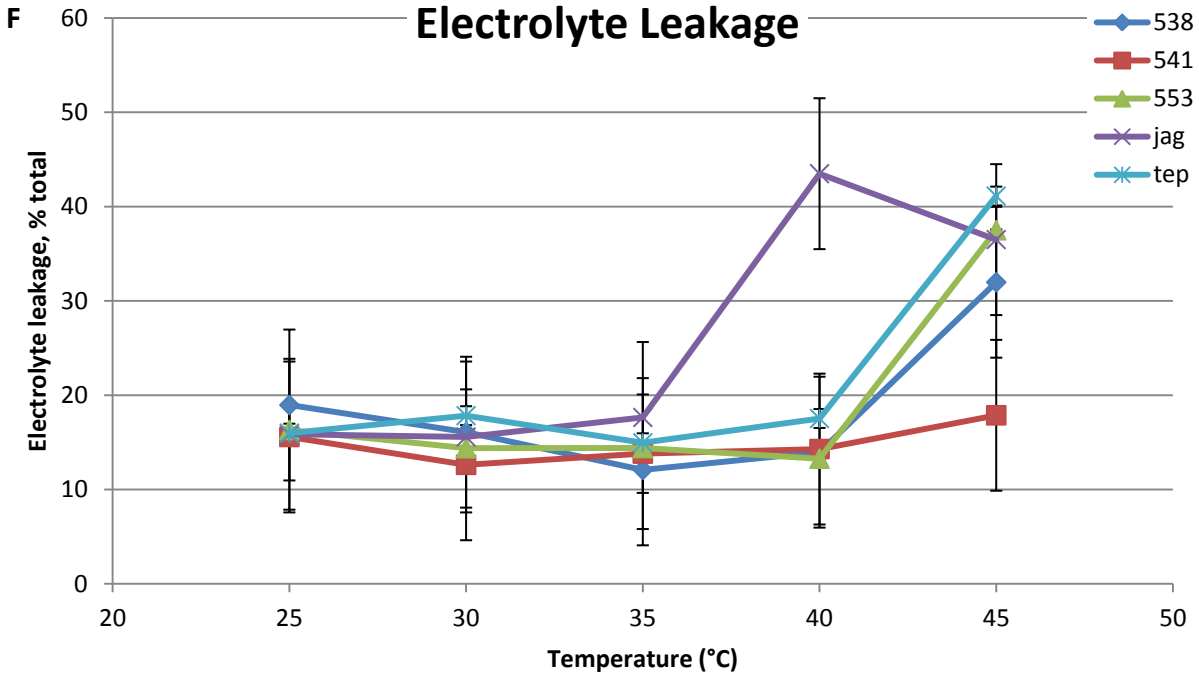

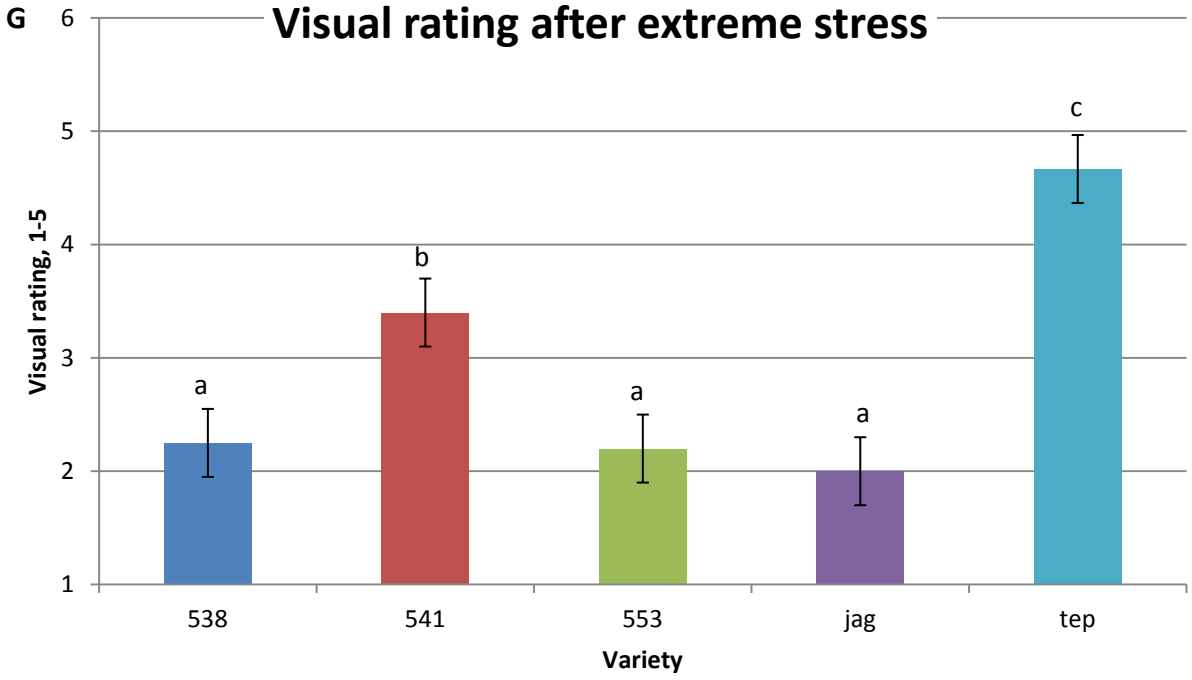

until the 45 °C period (Figure 15D). At that temperature, all except tepary saw a large increase in NPQ. Tepary was the exception at 45 °C for gas exchange and chlorophyll fluorescence measurements. It had higher values than the other genotypes for photosynthesis, stomatal conductance, and Φ_{PSII} and a lower NPQ at 45 °C.

TBARS concentrations were unchanging for all genotypes from 25-40 °C, but at 45 °C, TBARS increased for all genotypes (Figure 15E). Likewise, electrolyte leakage was stable for most genotypes until 45 °C when values for this parameter more than doubled for most genotypes (Figure 15F). I14541 had lower TBARS and electrolyte leakage values at 45 °C than the other genotypes.

For the final qualitative visual ratings, tepary scored significantly higher than the other genotypes, appearing undamaged on average, and I14541 scored higher than the other three common bean genotypes because it showed fewer signs of damage from heat stress (Figure 15G). I14538, I14553, and Jaguar appeared greatly damaged on average and had similarly low visual scores.

Discussion

Significance of different measures of stress

Heat stress affected most of the measured parameters, but heat stress did not become apparent until the most extreme temperatures tested. For example, photosynthesis was not appreciably reduced by heat stress until 45 °C (Figures 14A and 15A). This stands in contrast with a previous study that showed bean plants experiencing significant reductions in photosynthesis at 40 °C (Pimentel et al., 2013), but different genotypes and environmental

conditions were used in that study; most significantly, it lacked a gradual increase in the ambient air temperature. The decline in photosynthesis at these high temperatures was likely the result of a decrease in the specificity of rubisco for CO_2 compared to O_2, the deactivation of rubisco, and a decline in electron transport capacity (Haldimann and Feller, 2004; June et al., 2004; Sharkey, 2005; Sage and Kubien, 2007). Conversely, stomatal conductance increased with increasing temperature until it sharply declined at 45 °C (Figures 14B and 15B). From 25-40 °C, the increase in stomatal conductance could represent a stress resistance response that allows plants to maintain lower leaf temperatures via the transfer of latent heat. It is not likely that CO_2 concentration was limiting photosynthesis under these conditions, so stomatal conductance increased without a concomitant increase in photosynthesis; usually, the two processes correlate because of their regulation of each other (Wong et al., 1979).

The chlorophyll fluorescence data combined with the gas exchange data give a more complete picture of the response of the bean plants to increasing temperatures. From 25 to 40 °C, photosynthesis, stomatal conductance, and Φ_{PSII} were all stable and relatively high, indicating that the photosynthetic system was relatively unstressed. Stomatal conductance was high and so allowed a greater influx of CO_2 to maintain the high photosynthetic rates, and the high Φ_{PSII} indicated that most of the light energy absorbed was being partitioned towards fixing carbon (Baker, 2008); NPQ, a measure of excess energy being dissipated by various mechanisms (Kaiser et al., 2015), was low. At 45 °C, the photosynthetic systems of most genotypes experienced stress. The stress damaged components of the photosynthetic system and lowered photosynthetic rates; additionally, less absorbed light energy was being used by the damaged photosynthetic apparatus, so more energy was dissipated or quenched by other mechanisms, causing a decrease in Φ_{PSII} and an increase in NPQ. Finally, because photosynthesis was lower,

the stomata closed more to decrease conductance and match the influx of CO_2 with rate of CO_2 consumption.

Leaf canopy temperature showed little variation among genotypes especially when considering the large variation within a genotype (Figure 14E). Still, at an air temperature of 45 °C and with sufficient water, all genotypes lowered their leaf canopy temperatures to 38 to 41 °C. While plants rarely have canopy temperatures less than the air temperature in field settings, in this experiment, the low radiant energy (400 µmol photons m^{-2} s^{-1}) in the chamber and the well-watered condition of the plants allowed them to attain such low temperatures. In high temperature, high light conditions, many plants require the latent heat loss provided by high stomatal conductances to prevent damage by heat stress (Beerling et al., 2001; Schymanski et al., 2013). Additionally, plants with smaller leaves have smaller boundary layers and thus greater sensible heat exchange with the surrounding air (Okajima et al., 2012). Of the fifteen bean genotypes tested, tepary had noticeably smaller leaves, and the greater sensible heat loss enabled by such leaves would confer an advantage in hot desert environments, environments similar to those where tepary originated (Nabhan and Felger, 1978). Tepary's advantages in photosynthesis and stomatal conductance translate to productive benefits as well; a previous heat stress study showed that common bean's plant dry weight was decreased at elevated temperatures while tepary's weight was unchanged (Lin and Markhart, 1996). Considering all genotypes, stomatal conductance and canopy temperature correlated strongly with each other; if a genotype had a higher than average stomatal conductance, it also tended to have a lower than average canopy temperature (Table 6).

Insights from the focused experiment

The fifteen-genotype-screen indicated that the plants were experiencing heat stress only at 45 °C, so the smaller screen using five genotypes was performed to confirm and more narrowly identify at what point heat stress occurs. In this more focused experiment ranging from 25-45 °C, no measure showed consistent signs of stress until the air temperature reached 45 °C, and this observation was true for measures of gas exchange (Figure 15A-B), chlorophyll fluorescence (Figure 15C-D), and biochemical damage (Figure 15E-F). Duration of exposure is also a factor in heat stress; Udomprasert et al. (1995) found few differences between tepary and common bean when they were exposed to 40 or 45 °C for 6 hours. The current study suggests 24-48 hours are necessary to see the effects of heat stress.

Some slight differences in a genotype's visual rating were seen between the fifteen-genotype-screen and the smaller screen experiments (Figures 14F and 15G). These differences likely resulted from growing conditions of each experiment: because of space restrictions, the fifteen genotype experiment was grown to maturity in a greenhouse setting and only spent one week in a growth chamber, but the smaller experiment was grown to maturity in the growth chambers themselves. However, this change only made I14538 have a slightly lower visual rating and I14541 have a higher one in the smaller experiment.

Also of interest in the smaller experiment was that tepary's photosynthesis, stomatal conductance, Φ_{PSII}, and NPQ were least affected by 45 °C temperatures (Figure 15A-D) while I14541's TBARS and electrolyte leakage were least affected at this temperature (Figure 15E-F), and both genotypes looked the least damaged and had the highest visual ratings at the end of the experiment (Figure 15G). These results suggest two different heat tolerance strategies. Tepary's heat-capable photosynthetic machinery allowed it withstand high temperatures and shunt most of

its absorbed energy towards photosynthesis while I14541, despite having more a heat-compromised photosynthesis, was able to regulate reactive oxygen signaling and prevent damage to its cellular membranes, whose fluidity, permeability, and interactions with proteins are all affected by heat stress (Havaux, 1996; Pastenes and Horton, 1996; Bukhov et al., 1999).

Genotype performance in all measures of stress

For the fifteen-genotype-screen, some parameters correlated with each other in mechanistically expected ways: stomatal conductance and canopy temperature were negatively correlated with each other, Φ_{PSII} and NPQ were negatively correlated with each other, and Φ_{PSII} and visual rating were positively correlated with each other (Table 6). When examined individually, some genotypes had interesting trends across all parameters. Using genotype I14550 as an example, if the genotypes were ranked, it always placed in the bottom half, often the bottom fifth, for all measurements at 45 °C: it had low photosynthesis, stomatal conductance, and Φ_{PSII}, and it had high NPQ and canopy temperature. Consequently, it showed signs of damage to its leaves and had a low visual rating as well. Conversely, tepary ranked high for all of these measurements and had a high visual rating. Some exceptions do exist: SER-95 had low photosynthesis and conductance but high Φ_{PSII} and low NPQ, and it ended up having a high score for visual rating. The qualitative visual rating was an excellent indicator of leaf damage caused by heat stress and correlated well with other measures (Table 6).

However, these parameters did not have a strong connection to a genotype's level of drought tolerance in the field. Drought tolerant genotypes such as tepary, SER-95, I14538, and I14549 did have the highest visual ratings of the fifteen genotypes, but then drought tolerant genotypes such as I14553 and I14544 ranked relatively low in terms of their visual rating (Figure

14F). This weaker connection could have been the result of a variety of factors. For all the genotypes labeled as a number, their characterization comes from a single drought field test performed in Mayaguez, Puerto Rico (Tim Porch, personal communication). Under both drought and nonstress conditions, common bean yield can vary greatly in different years and locations (Terán and Singh, 2002*b*; Muñoz-Perea et al., 2006; Beebe et al., 2008; Builes et al., 2011), so further field tests in different locations and years could create a more accurate characterization of each genotype's drought tolerance that escapes the current study that relies on a single field trial.

The other factor contributing to the discrepancy is that heat and drought stress are two distinct abiotic stresses with different response pathways (Mittler, 2006), so it is possible for a genotype to be tolerant of one and not the other. While heat and drought stress share a number of transcriptional response and redox-signaling pathways (Swindell et al., 2007; Foyer and Noctor, 2009), much like any stress, heat and drought cause distinctly different changes in the transcriptome and metabolome (Shulaev et al., 2008; Rasmussen et al., 2013), and the combination of heat and drought stress produce a third, unique response as well (Rizhsky et al., 2002, 2004). Heat, drought, and the combination of the two share relatively few genes in their response pathways compared to the number they do not share (Rizhsky et al., 2004). Thus, depending on the alleles found in each pathway, it is possible for a genotype to be resistant to only drought stress, only heat stress, or both. Some of the genotypes that performed well under the drought-stressed field trial may have performed poorly in this heat stress screen simply because they are drought tolerant but not heat tolerant. The heat-screening method was useful in characterizing germplasm, especially in identifying some of the most tolerant genotypes, but it should not be relied upon exclusively and its results should be considered alongside the results of several other methods. Although based on the high coincidence of heat and drought stress in

several bean production areas, breeders may not want a drought tolerant cultivar if it cannot also withstand heat (Araújo et al., 2014), so a consideration of combined stresses may make the heat screening method a more attractive tool.

Conclusions

Under these controlled conditions, the bean genotypes did not experience significant heat stress until air temperatures were increased to 45 °C. This temperature was sufficient to affect photosynthesis, stomatal conductance, Φ_{PSII}, NPQ, electrolyte leakage, and TBARS and to cause visible damage to plant leaves. For a single genotype, the degree of change in one of these measures was often well-correlated with the degree of change in another. Because of this correlation, any or all of these measurements would be appropriate in other screens of germplasm using heat stress. Even an experimenter limited by time or available equipment could use one or two measures and still perform a meaningful screen. The stress tolerant, related species tepary bean outperformed all common bean genotypes for most measures of heat tolerance, and known drought-tolerant common bean genotype SER-95 performed better than most other genotypes as well. As would be expected, the correlation between drought tolerance in the field and heat tolerance under controlled conditions was not perfect because great differences exist between the two environments and drought and heat tolerance share only some of their pathways; however, the screen still identified the more tolerant genotypes with few false positives. Used in conjunction with field data and other tests, heat screening is a simple and quick method of characterizing large groups of germplasm.

Chapter 5: Conclusions and recommendations

The research of this dissertation adds to an understanding of stress-related physiology in common bean and tepary bean. While proline had little response to drought stress in beans and likely plays no role in acclimation to drought, certain organic acids and sugars such as malic acid, glucose, fructose, inositol, and raffinose significantly increased in response to drought stress. These soluble carbohydrates accumulated in sufficient quantities to contribute to the osmotic adjustment of plant tissues, and drought tolerant bean genotypes accumulated a higher concentration of these compounds than susceptible genotypes. Supporting these results, the same genotypes with higher concentrations of soluble carbohydrates also had lower leaf water potentials under drought stress, but no significant differences in leaf water potential were found among genotypes in the control treatment. The differences among genotypes were the result of drought stress and were not constitutive, for all genotypes had similar concentrations of soluble carbohydrates under control conditions. Whether as osmolytes, osmoprotectants, or as signaling factors, these soluble carbohydrates are an important part of stress response in beans. While measuring individual sugar concentrations would be onerous for a plant breeder who has to evaluate large quantities of germplasm, it could be effectively used on a smaller scale to evaluate a smaller group of genotypes being considered as parents in future crosses. Furthermore, in field trials that experience a period of drought stress, leaf water potential measurements could be taken on a moderate number of genotypes, perhaps those of special interest, to test for the degree of osmotic adjustment and thus indirectly for accumulation of sugars.

Despite being an integral stress-signaling hormone, abscisic acid (ABA) concentrations under drought stress had little correlation with drought tolerance. Under the most severe drought stress, the most susceptible genotype had the highest concentration of ABA in leaf tissue. Such high ABA concentrations probably do not result in drought susceptibility *per se*, but rather they

reflect the level of stress a plant is experiencing, so high ABA concentrations in bean plants result from the failure of other mechanisms to mitigate the effects of drought stress. ABA is one of the prime regulators of stomatal aperture, and the gas exchange data from stressed plants revealed characteristics of drought tolerance in beans. Drought tolerant genotypes had lower stomatal conductances under both control and drought stressed conditions and consequently tended to have lower photosynthetic rates as well. Factors downstream of ABA signaling could be more sensitive to ABA and thus result in lower stomatal conductances in tolerant genotypes, even when their concentrations of ABA are similar to susceptible genotypes. The exogenous ABA experiment showed that only one of the two tolerant genotypes displayed increased sensitivity, so other factors determining stomatal conductance must exist in beans. That tolerant genotypes also had lower stomatal conductances under control conditions, when ABA is not a contributing factor, supports the existence of other mechanisms, mechanisms which are perhaps constitutive. Based on grafting experiments, whatever mechanisms that do control stomatal conductance are located in shoot tissues and not root tissues. A following experiment found stomatal density is not the factor behind differences in stomatal conductance as two genotypes with large contrasts in phylogeny and drought tolerance had the same density of stomata. Future research should investigate further the nature of the differences in stomatal conductance between susceptible and tolerant genotypes, for it seems to be key to drought tolerance in beans. Breeding for reduced stomatal conductances should be an easier goal than some because it is a constitutive trait that does not require drought stress to be measureable. Of course, the tradeoff between lower conductances and lower photosynthetic rates must also be considered, and extreme selection for this trait could result in reduced productivity. Based on this tradeoff and the time required to take measurements, selecting for stomatal conductance should be done at more

advanced stages of the breeding cycle when productivity is already established and there are fewer lines to evaluate.

When doing heat stress experiments, it is essential that sufficiently high temperatures are used and that duration of exposure is long enough to induce stress. For the bean genotypes used in the heat screening experiment, 45 °C for two days served to induce measureable stress effects. The different measures of physiology and stress correlated well with each other, so any one of them could be used in similar screening experiments. Overall, the genotypes' heat tolerance and drought tolerance were not perfectly correlated with each other, but it is interesting to note that the genotypes that displayed the greatest heat tolerance in the screen were among the most drought tolerant in the field. Heat and drought stress are often coincident in field settings, so having some degree of heat tolerance contributes to better tolerance of most drought events as well. Additionally, now that the basic heat screening method described in Chapter 4 was shown to have some utility in beans, researchers can conduct experiments to tweak the method to screen for the more technically challenging stress of drought or even combined drought and heat stress. Breeders can then use these screening methods in the off-season to add an extra layer of characterization to lines also being evaluated in the field.

BIBLIOGRAPHY

BIBLIOGRAPHY

Acosta-Gallegos J, White JW. 1995. Phenological plasticity as an adaptation by common bean to rainfed environments. Crop Science **35**, 199–204.

Akibode S, Maredia M. 2011. Global and Regional Trends in Production, Trade and Consumption of Food Legume Crops. CGIAR.

Allen DJ, Ort DR. 2001. Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends in Plant Science **6**, 36–42.

Araújo SS, Beebe S, Crespi M, *et al.* 2014. Abiotic stress responses in legumes: strategies used to cope with environmental challenges. Critical Reviews in Plant Sciences **34**, 237–280.

Araus JL, Slafer GA, Reynolds MP, Royo C. 2002. Plant breeding and drought in C3 cereals: what should we breed for? Annals of Botany **89**, 925–940.

Asada K. 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiology **141**, 391–396.

Asch F, Dörffling K, Dingkuhn M. 1995. Response of rice varieties to soil salinity and air humidity: A possible involvement of root-borne ABA. Plant and Soil **177**, 11–19.

Asfaw A, Blair M. 2012. Quantitative trait loci for rooting pattern traits of common beans grown under drought stress versus non-stress conditions. Molecular Breeding **30**, 681–695.

Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J. 2007. A central integrator of transcription networks in plant stress and energy signalling. Nature **448**, 938–942.

Bahieldin A, Mahfouz HT, Eissa HF, Saleh OM, Ramadan AM, Ahmed IA, Dyer WE, El-Itriby HA, Madkour MA. 2005. Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. Physiologia Plantarum **123**, 421–427.

Bailey NJC, Oven M, Holmes E, Nicholson JK, Zenk MH. 2003. Metabolomic analysis of the consequences of cadmium exposure in Silene cucubalus cell cultures via H-1 NMR spectroscopy and chemometrics. Phytochemistry **62**, 851–858.

Baker NR. 2008. Chlorophyll fluorescence: A probe of photosynthesis in vivo. Annual Review of Plant Biology **59**, 89–113.

Barchet GLH, Dauwe R, Guy RD, Schroeder WR, Soolanayakanahally RY, Campbell MM, Mansfield SD. 2014. Investigating the drought-stress response of hybrid poplar genotypes by metabolite profiling. Tree Physiology **34**, 1203–1219.

Bates L, Waldren R, Teare I. 1973. Rapid determination of free proline for water stress studies. Plant and Soil **39**, 205–207.

Battisti DS, Naylor RL. 2009. Historical warnings of future food insecurity with unprecedented seasonal heat. Science **323**, 240–244.

Beebe S. 2012. Common Bean Breeding in the Tropics. Plant Breeding Reviews. John Wiley & Sons, Inc., 357–426.

Beebe S, Ramirez J, Jarvis A, Rao IM, Mosquera G, Bueno JM, Blair MW. 2011. Genetic Improvement of Common Beans and the Challenges of Climate Change. Yadav SS, Redden RJ, Hatfield JL, Lotze-Campen H, Hall AE, eds. Crop Adaptation to Climate Change. Wiley-Blackwell, 356–369.

Beebe S, Rao I, Blair M, Acosta J. 2013. Phenotyping common beans for adaptation to drought. Frontiers in Physiology **4**, 347-362.

Beebe SE, Rao IM, Cajiao C, Grajales M. 2008. Selection for drought resistance in common bean also improves yield in phosphorus limited and favorable environments. Crop Science **48**, 582–592.

Beebe S, Rao I, Mukankusi C, Buruchara R. 2012. Improving resource use efficiency and reducing risk of common bean production in Africa, Latin America, and the Carribean. Eco-Efficiency: From Vision to Reality. CIAT.

Beerling DJ, Osborne CP, Chaloner WG. 2001. Evolution of leaf-form in land plants linked to atmospheric CO2 decline in the Late Palaeozoic era. Nature **410**, 352–354.

Bielenberg D, Miller J, Berg V. 2003. Paraheliotropism in two Phaseolus species: combined effects of photon flux density and pulvinus temperature, and consequences for leaf gas exchange. Environmental and Experimental Botany **49**, 95–105.

Bitocchi E, Nanni L, Bellucci E, *et al.* 2012. Mesoamerican origin of the common bean (Phaseolus vulgaris L.) is revealed by sequence data. Proceedings of the National Academy of Sciences **109**, E788–E796.

Blair M, Fernandez A, Ishitani M, Moreta D, Seki M, Ayling S, Shinozaki K. 2011. Construction and EST sequencing of full-length, drought stress cDNA libraries for common beans (Phaseolus vulgaris L.). BMC Plant Biology **11**, 171.

Blair MW, Galeano CH, Tovar E, Munoz Torres MC, Velasco Castrillon A, Beebe SE, Rao IM. 2012. Development of a Mesoamerican intra-genepool genetic map for quantitative trait loci detection in a drought tolerant x susceptible common bean (Phaseolus vulgaris L.) cross. Molecular Breeding **29**, 71–88.

Blair, MW, Soler, A, Cortés, AJ. 2012. Diversification and population structure in common beans (Phaseolus vulgaris L.). PLoS ONE **7**, e49488.

Blatt M, Armstrong F. 1993. K+ channels of stomatal guard cells: abscisic acid evoked control of the outward rectifier mediated by cytoplasmic pH. Planta **191**, 330–341.

Blum A. 2011. Breeding Considerations and Strategies.

Boutraa T, Sanders FE. 2001. Influence of water stress on grain yield and vegetative growth of two cultivars of bean (Phaseolus vulgaris L.). Journal of Agronomy & Crop Science **187**, 251.

Bowes G, Ogren WL, Hageman RH. 1971. Phosphoglycolate production catalyzed by ribulose diphosphate carboxylase. Biochemical and Biophysical Research Communications **45**, 716–722.

Boyer JS, Westgate ME. 2004. Grain yields with limited water. Journal of Experimental Botany **55**, 2385–2394.

Boyle MG, Boyer JS, Morgan PW. 1991. Stem infusion of liquid culture medium prevents reproductive failure of maize at low water potential. Crop Science **31**, 1246–1252.

Broughton WJ, Hernández G, Blair M, Beebe S, Gepts P, Vanderleyden J. 2003. Beans (Phaseolus spp.) – model food legumes. Plant and Soil **252**, 55–128.

Buckley TN, Mott KA. 2013. Modelling stomatal conductance in response to environmental factors. Plant Cell and Environment **36**, 1691–1699.

Builes VHR, Porch TG, Harmsen EW. 2011. Genotypic differences in water use efficiency of common bean under drought stress. Agronomy Journal **103**, 1206–1215.

Bukhov N, Wiese C, Neimanis S, Heber U. 1999. Heat sensitivity of chloroplasts and leaves: leakage of protons from thylakoids and reversible activation of cyclic electron transport. Photosynthesis Research **59**, 81–93.

Butare L, Rao I, Lepoivre P, Polania J, Cajiao C, Cuasquer J, Beebe S. 2011. New genetic sources of resistance in the genus Phaseolus to individual and combined aluminium toxicity and progressive soil drying stresses. Euphytica **181**, 385–404.

von Caemmerer S, Evans JR. 2014. Temperature responses of mesophyll conductance differ greatly between species. Plant, Cell & Environment, n/a–n/a.

Carrari F, Fernie AR, Iusem ND. 2004. Heard it through the grapevine? ABA and sugar cross-talk: the ASR story. Trends in Plant Science **9**, 57–59.

Casati P, Drincovich M, Edwards G, Andreo C. 1999. Malate metabolism by NADP-malic enzyme in plant defense. Photosynthesis Research **61**, 99–105.

Castonguay Y, Markhart A. 1991. Saturated rates of photosynthesis in water-stressed leaves of common bean and tepary bean. Crop Science **31**, 1605–1611.

Castonguay Y, Markhart A. 1992. Leaf gas-exchange in water-stressed common bean and tepary bean. Crop Science **32**, 980–986.

Cattivelli L, Rizza F, Badeck F-W, Mazzucotelli E, Mastrangelo AM, Francia E, Mare C, Tondelli A, Stanca AM. 2008. Drought tolerance improvement in crop plants: An integrated view from breeding to genomics. Field Crops Research **105**, 1–14.

Cavalieri A, Merchant A, van Volkenburgh E. 2011. Why not beans? Foreword. Functional Plant Biology **38**, III–VI.

Centritto M, Loreto F, Chartzoulakis K. 2003. The use of low [CO2] to estimate diffusional and non-diffusional limitations of photosynthetic capacity of salt-stressed olive saplings. Plant, Cell & Environment **26**, 585–594.

CGIAR. 2015. Developing Beans that Can Beat the Heat.

Chaves M. 1991. Effects of water deficits on carbon assimilation. Journal of Experimental Botany **42**, 1–16.

Chaves M, Maroco J, Pereira J. 2003. Understanding plant responses to drought - from genes to the whole plant. Functional Plant Biology **30**, 239–264.

Chaves MM, Oliveira MM. 2004. Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. Journal of Experimental Botany **55**, 2365–2384.

Chen THH, Murata N. 2008. Glycinebetaine: an effective protectant against abiotic stress in plants. Trends in Plant Science **13**, 499–505.

Christmann A, Weiler EW, Steudle E, Grill E. 2007. A hydraulic signal in root-to-shoot signalling of water shortage. The Plant Journal **52**, 167–174.

Cornic G, Le Gouallec J-L, Briantais JM, Hodges M. 1989. Effect of dehydration and high light on photosynthesis of two C3 plants (Phaseolus vulgaris L. and Elatostema repens (Lour.) Hall f.). Planta **177**, 84–90.

Cortés, AJ, Monserrate, FA, Ramírez-Villegas, J, Madriñán, S, Blair, MW. 2013. Drought tolerance in wild plant populations: the case of common beans (Phaseolus vulgaris L.). PLoS ONE **8**, e62898.

Cutler A, Krochko J. 1999. Formation and breakdown of ABA. Trends in Plant Science **4**, 472–478.

Cutler SR, Rodriguez PL, Finkelstein RR, Abrams SR. 2010. Abscisic acid: emergence of a core signaling network. Merchant, S and Briggs, WR and Ort, D eds. Annual Review of Plant Biology. Annual Review of Plant Biology **61,** 651–679.

Dalal M, Inupakutika M. 2014. Transcriptional regulation of ABA core signaling component genes in sorghum (Sorghum bicolor L. Moench). Molecular Breeding **34**, 1517–1525.

Damour G, Simonneau T, Cochard H, Urban L. 2010. An overview of models of stomatal conductance at the leaf level. Plant Cell and Environment **33**, 1419–1438.

Daszkowska-Golec A, Szarejko I. 2013. Open or close the gate – stomata action under the control of phytohormones in drought stress conditions. Frontiers in Plant Science **4**.

Delfine S, Alvino A, Villani MC, Loreto F. 1999. Restrictions to carbon dioxide conductance and photosynthesis in spinach leaves recovering from salt stress. Plant Physiology **119**, 1101–1106.

Demmig-Adams B, Adams W. 1996. The role of xanthopyll cycle carotenoids in the protection of photosynthesis. Trends in Plant Science **1**, 21–26.

Desikan R, Griffiths R, Hancock J, Neill S. 2002. A new role for an old enzyme: nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in Arabidopsis thaliana. Proceedings of the National Academy of Sciences **99**, 16314–16318.

Devi MJ, Sinclair TR, Beebe SE, Rao IM. 2013. Comparison of common bean (Phaseolus vulgaris L.) genotypes for nitrogen fixation tolerance to soil drying. Plant and Soil **364**, 29–37.

Doubnerová V, Ryšlavá H. 2011. What can enzymes of C4 photosynthesis do for C3 plants under stress? Plant Science **180**, 575–583.

Drake PL, Froend RH, Franks PJ. 2012. Smaller, faster stomata: scaling of stomatal size, rate of response, and stomatal conductance. Journal of Experimental Botany **64**, 549-505.

Easlon HM, Bloom AJ. 2014. Easy Leaf Area: Automated Digital Image Analysis for Rapid and Accurate Measurement of Leaf Area. Applications in Plant Sciences **2**, 421-428.

Ebell LF. 1969. Specific total starch determinations in conifer tissues with glucose oxidase. Phytochemistry **8**, 25–36.

Edwards S, Nguyen B-T, Do B, Roberts JKM. 1998. Contribution of malic enzyme, pyruvate kinase, phosphoenolpyruvate carboxylase, and the krebs cycle to respiration and biosynthesis and to intracellular pH regulation during hypoxia in maize root tips observed by nuclear magnetic resonance imaging and gas chromatography-mass spectrometry. Plant Physiology **116**, 1073–1081.

Ehleringer J, Klassen S, Clayton C, Sherril D, Fullerholbrook M, Fu Q, Cooper T. 1991. Carbon isotope discrimination and transpiration efficiency in common bean. Crop Science **31**, 1611–1615.

Ehleringer J, Pearcy RW. 1983. Variation in quantum yield for CO_2 uptake among C_3 and C_4 plants. Plant Physiology **73**, 555–559.

Escobar-Gutierrez A, Zipperlin B, Carbonne F, Moing A, Gaudillere J. 1998. Photosynthesis, carbon partitioning and metabolite content during drought stress in peach seedlings. Australian Journal of Plant Physiology **25**, 197–205.

Evans J, Sharkey T, Berry J, Farquhar G. 1986. Carbon isotope discrimination measured concurrently with gas exchange to investigate CO2 diffusion in leaves of higher plants. Australian Journal of Plant Physiology **13**, 281–292.

FAOSTAT. 2014. FAOSTAT.

Fischer RA, Rees D, Sayre KD, Lu Z-M, Condon AG, Saavedra AL. 1998. Wheat yield progress associated with higher stomatal conductance and photosynthetic rate, and cooler canopies. Crop Science **38**, 1467–1475.

Fletcher RJ, Bell IP, Lambert JP. 2004. Public health aspects of food fortification: a question of balance. Proceedings of the Nutrition Society **63**, 605–614.

Flexas J, Bota J, Escalona JM, Sampol B, Medrano H. 2002. Effects of drought on photosynthesis in grapevines under field conditions: an evaluation of stomatal and mesophyll limitations. Functional Plant Biology **29**, 461–471.

Flexas J, Bota J, Loreto F, Cornic G, Sharkey T. 2004. Diffusive and metabolic limitations to photosynthesis under drought and salinity in C3 plants. Plant Biology **6**, 269–279.

Foyer CH, Noctor G. 2009. Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications. Antiooxidants & Redox Signaling **11**, 861–905.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiology **155**, 2–18.

Frahm M, Rosas J, Mayek-Perez N, Lopez-Salinas E, Acosta-Gallegos J, Kelly J. 2004. Breeding beans for resistance to terminal drought in the lowland tropics. Euphytica **136**, 223–232.

Frébort I, Kowalska M, Hluska T, Frébortová J, Galuszka P. 2011. Evolution of cytokinin biosynthesis and degradation. Journal of Experimental Botany **62**, 2431–2452.

Frensch J, Hsiao TC. 1994. Transient responses of cell turgor and growth of maize Roots as affected by changes in water potential. Plant Physiology **104**, 247–254.

Gagneul D, Aienouche A, Duhaze C, Lugan R, Larher FR, Bouchereau A. 2007. A reassessment of the function of the so-called compatible solutes in the halophytic Plumbaginaceae Limonium latifolium. Plant Physiology **144**, 1598–1611.

Garcia A, Engler J, Iyer S, Gerats T, VanMontagu M, Caplan A. 1997. Effects of osmoprotectants upon NaCl stress in rice. Plant Physiology **115**, 159–169.

Geigenberger P, Reimholz R, Geiger M, Merlo L, Canale V, Stitt M. 1997. Regulation of sucrose and starch metabolism in potato tubers in response to short-term water deficit. Planta **201**, 502–518.

Geitmann A, Ortega JKE. 2009. Mechanics and modeling of plant cell growth. Trends in Plant Science **14**, 467–478.

Gengenbach BG. 1977. Development of maize caryopses resulting from in-vitro pollination. Planta **134**, 91–93.

Gepts P, Bliss FA. 1985. F1 hybrid weakness in the common bean: Differential geographic origin suggets two gene pools in cultivated bean germplasm. Journal of Heredity **76**, 447–450.

Graham RD, Welch RM, Saunders DA, *et al.* 2007. Nutritious subsistence food systems. Donald L. Sparks ed. Advances in Agronomy. Academic Press, 1–74.

Gross Y, Kigel J. 1994. Differential sensitivity to high temperature of stages in the reproductive development of common bean (Phaseolus vulgaris L.). Field Crops Research **36**, 201–212.

Guo P, Baum M, Grando S, Ceccarelli S, Bai G, Li R, von Korff M, Varshney RK, Graner A, Valkoun J. 2009. Differentially expressed genes between drought-tolerant and drought-sensitive barley genotypes in response to drought stress during the reproductive stage. Journal of Experimental Botany **60**, 3531–3544.

Gupta A, Kaur N. 2005. Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. Journal of Biosciences **30**, 761–776.

Haldimann P, Feller U. 2004. Inhibition of photosynthesis by high temperature in oak (Quercus pubescens L.) leaves grown under natural conditions closely correlates with a reversible heat-dependent reduction of the activation state of ribulose-1,5-bisphosphate carboxylase/oxygenase. Plant, Cell & Environment **27**, 1169–1183.

Harb A, Krishnan A, Ambavaram MMR, Pereira A. 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. Plant Physiology **154**, 1254–1271.

Hare PD, Cress WA. 1997. Metabolic implications of stress-induced proline accumulation in plants. Plant Growth Regulation **21**, 79–102.

Hare PD, Cress WA, Van Staden J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant, Cell & Environment **21**, 535–553.

Hartung W, Sauter A, Hose E. 2002. Abscisic acid in the xylem: where does it come from, where does it go to? Journal of Experimental Botany **53**, 27–32.

Hauser F, Waadt R, Schroeder JI. 2011. Evolution of abscisic acid synthesis and signaling mechanisms. Current Biology **21**, R346–R355.

Havaux M. 1992. Stress tolerance of photosystem II in vivo: antagonistic effects of water, heat, and photoinhibition stresses. Plant Physiology **100**, 424–432.

Havaux M. 1996. Short-term responses of photosystem I to heat stress. Photosynthesis Research **47**, 85–97.

Héroult A, Lin Y-S, Bourne A, Medlyn B, Ellsworth D. 2013. Optimal stomatal conductance in relation to photosynthesis in climatically contrasting Eucalyptus species under drought.

Hetherington AM, Woodward FI. 2003. The role of stomata in sensing and driving environmental change. Nature **424**, 901–908.

Hoekstra FA, Golovina EA, Buitink J. 2001. Mechanisms of plant desiccation tolerance. Trends in Plant Science **6**, 431–438.

Holbrook N, Shashidhar V, James R, Munns R. 2002. Stomatal control in tomato with ABA-deficient roots: response of grafted plants to soil drying. Journal of Experimental Botany **53**, 1503–1514.

Hommel R, Siegwolf R, Saurer M, Farquhar GD, Kayler Z, Ferrio JP, Gessler A. 2014. Drought response of mesophyll conductance in forest understory species – impacts on water-use efficiency and interactions with leaf water movement. Physiologia Plantarum **152**, 98–114.

Hoque MA, Banu MNA, Okuma E, Murata Y. 2007. Exogenous proline and glycinebetaine increase NaCl-induced ascorbate-glutathione cycle enzyme activities, and proline improves salt tolerance more than glycinebetaine in tobacco Bright Yellow-2 suspension-cultured cells. Journal of Plant Physiology **164**, 1457–1468.

Hossain MA, Munemasa S, Uraji M, Nakamura Y, Mori IC, Murata Y. 2011. Involvement of endogenous abscisic acid in methyl jasmonate-induced stomatal closure in Arabidopsis. Plant Physiology **156**, 430–438.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. Annual Review of Plant Physiology and Plant Molecular Biology **47**, 377–403.

Izquierdo J, Hosfield G. 1983. The relationship of seed filling to yield among dry beans with differing architectural forms. Journal of the American Society for Horticultural Science **108**, 106–111.

Ji X, Dong B, Shiran B, Talbot MJ, Edlington JE, Hughes T, White RG, Gubler F, Dolferus R. 2011. Control of abscisic acid catabolism and abscisic acid homeostasis is important for reproductive stage stress tolerance in cereals. Plant Physiology **156**, 647–662.

Jordan D, Ogren W. 1984. The CO2/O2 specificity of ribulose 1,5-bisphosphate carboxylase/oxygenase. Planta **161**, 308–313.

June T, Evans JR, Farquhar GD. 2004. A simple new equation for the reversible temperature dependence of photosynthetic electron transport: a study on soybean leaf. Functional Plant Biology **31**, 275–283.

Kaiser E, Morales A, Harbinson J, Kromdijk J, Heuvelink E, Marcelis LFM. 2015. Dynamic photosynthesis in different environmental conditions. Journal of Experimental Botany **66**, 2415–2426.

Kakumanu A, Ambavaram MMR, Klumas C, Krishnan A, Batlang U, Myers E, Grene R, Pereira A. 2012. Effects of drought on gene expression in maize reproductive and leaf meristem tissue revealed by RNA-seq. Plant Physiology **160**, 846–867.

Kanno Y, Jikumaru Y, Hanada A, Nambara E, Abrams SR, Kamiya Y, Seo M. 2010. Comprehensive hormone profiling in developing Arabidopsis seeds: examination of the site of ABA biosynthesis, ABA transport and hormone interactions. Plant and Cell Physiology **51**, 1988–2001.

Kebeish R, Niessen M, Thiruveedhi K, Bari R, Hirsch H-J, Rosenkranz R, Stabler N, Schonfeld B, Kreuzaler F, Peterhansel C. 2007. Chloroplastic photorespiratory bypass increases photosynthesis and biomass production in Arabidopsis thaliana. Nat Biotech **25**, 593–599.

Keller J, Loescher W. 1989. Nonstructural carbohydrate partitioning in perennial parts of sweet cherry. Journal of the American Society for Horticultural Science **114**, 969–975.

Kelly JD, Hosfield GL, Varner GV, Uebersax MA, Taylor J. 2001. Registration of 'Jaguar' black bean. Crop Science **41**, 1647–1648.

Kelly J, Miklas P. 1998. The role of RAPD markers in breeding for disease resistance in common bean. Molecular Breeding **4**, 1–11.

Kelly JD, Varner GV, O'Boyle P, Long B. 2009*a*. Registration of 'Zorro' black bean. Journal of Plant Registrations **3**, 226–230.

Kelly JD, Varner GV, Roman B, Long B. 2009*b*. Registration of 'Fuji' otebo bean. Journal of Plant Registrations **3**, 223–225.

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K. 2007. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. The Plant Journal **50**, 347–363.

Koch K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Current Opinion in Plant Biology **7**, 235–246.

Kornyeyev D, Logan BA, Allen RD, Holaday AS. 2005. Field-grown cotton plants with elevated activity of chloroplastic glutathione reductase exhibit no significant alteration of diurnal or seasonal patterns of excitation energy partitioning and CO2 fixation. Field Crops Research **94**, 165–175.

Kornyeyev D, Logan BA, Payton P, Allen RD, Holaday AS. 2001. Enhanced photochemical light utilization and decreased chilling-induced photoinhibition of photosystem II in cotton

overexpressing genes encoding chloroplast-targeted antioxidant enzymes. Physiologia Plantarum **113**, 323–331.

Kornyeyev D, Logan BA, Payton PR, Allen RD, Holaday AS. 2003. Elevated chloroplastic glutathione reductase activities decrease chilling-induced photoinhibition by increasing rates of photochemistry, but not thermal energy dissipation, in transgenic cotton. Functional Plant Biology **30**, 101–110.

Koussevitzky S, Suzuki N, Huntington S, Armijo L, Sha W, Cortes D, Shulaev V, Mittler R. 2008. Ascorbate peroxidase 1 plays a key role in the response of Arabidopsis thaliana to stress combination. Journal of Biological Chemistry **283**, 34197–34203.

Kozaki A, Takeba G. 1996. Photorespiration protects C3 plants from photooxidation. Nature **384**, 557–560.

Kudoyarova G, Veselova S, Hartung W, Farhutdinov R, Veselov D, Sharipova G. 2011. Involvement of root ABA and hydraulic conductivity in the control of water relations in wheat plants exposed to increased evaporative demand. Planta **233**, 87–94.

Levitt J. 1972. *Responses of plants to environmental stresses*. New York: Academic Press.

Lin T, Markhart A. 1996. Phaseolus acutifolius A. Gray is more heat tolerant than P-vulgaris L in the absence of water stress. Crop Science **36**, 110–114.

Liu S, Cheng Y, Zhang X, Guan Q, Nishiuchi S, Hase K, Takano T. 2007. Expression of an NADP-malic enzyme gene in rice (Oryza sativa. L) is induced by environmental stresses; over-expression of the gene in Arabidopsis confers salt and osmotic stress tolerance. Plant Molecular Biology **64**, 49–58.

Liu S, Yu K, Park SJ. 2009. Marker-assisted breeding for resistance to common bacterial blight in common bean. Huttunen N, Sinisalo T, eds. Plant Breeding. 211–226.

Lizana C, Wentworth M, Martinez J, *et al.* 2006. Differential adaptation of two varieties of common bean to abiotic stress - I. Effects of drought on yield and photosynthesis. Journal of Experimental Botany **57**, 685–697.

Lobell DB, Gourdji SM. 2012. The influence of climate change on global crop productivity. Plant Physiology **160**, 1686–1697.

Locke AM, Ort DR. 2014. Leaf hydraulic conductance declines in coordination with photosynthesis, transpiration and leaf water status as soybean leaves age regardless of soil moisture. Journal of Experimental Botany **65**, 6617–6627.

Loewus FA, Murthy PPN. 2000. myo-Inositol metabolism in plants. Plant Science **150**, 1–19.

Long S, Bernacchi C. 2003. Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. Journal of Experimental Botany **54**, 2393–2401.

Long S, Zhu X, Naidu S, Ort D. 2006. Can improvement in photosynthesis increase crop yields? Plant Cell and Environment **29**, 315–330.

MacRobbie EAC. 1998. Signal transduction and ion channels in guard cells. Philosophical Transactions of the Royal Society of London B: Biological Sciences **353**, 1475–1488.

Markhart AH III. 1985. Comparative water relations of Phaseolus vulgaris L. and Phaseolus acutifolius Gray. Plant Physiology **77**, 113–117.

Ma Y, Szostkiewicz I, Korte A, Moes D, Yang Y, Christmann A, Grill E. 2009. Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science **324**, 1064–1068.

McAinsh MR, Brownlee C, Hetherington AM. 1992. Visualizing changes in cytosolic-free Ca2+ during the response of stomatal guard cells to abscisic acid. The Plant Cell **4**, 1113–1122.

McClean PE, Burridge J, Beebe S, Rao IM, Porch TG. 2011. Crop improvement in the era of climate change: an integrated, multi-disciplinary approach for common bean (Phaseolus vulgaris). Functional Plant Biology **38**, 927–933.

Medrano H, Escalona J, Bota J, Gulias J, Flexas J. 2002. Regulation of photosynthesis of C-3 plants in response to progressive drought: Stomatal conductance as a reference parameter. Annals of Botany **89**, 895–905.

Mejía-Jiménez A, Muñoz C, Jacobsen HJ, Roca WM, Singh SP. 1994. Interspecific hybridization between common and tepary beans: increased hybrid embryo growth, fertility, and efficiency of hybridization through recurrent and congruity backcrossing. Theoretical and Applied Genetics **88**, 324–331.

Mencuccini M, Comstock J. 1999. Variability in hydraulic architecture and gas exchange of common bean (Phaseolus vulgaris) cultivars under well-watered conditions: interactions with leaf size. Australian Journal of Plant Physiology **26**, 115–124.

Mittler R. 2006. Abiotic stress, the field environment and stress combination. Trends in Plant Science **11**, 15–19.

Morgan JM. 1984. Osmoregulation and water stress in higher plants. Annual Review of Plant Physiology **35**, 299–319.

Mori IC, Murata Y. 2011. ABA signaling in stomatal guard cells: lessons from Commelina and Vicia. Journal of Plant Research **124**, 477–487.

Moroney J, Jungnick N, DiMario R, Longstreth D. 2013. Photorespiration and carbon concentrating mechanisms: two adaptations to high O2, low CO2 conditions. Photosynthesis Research **117**, 121–131.

Mukeshimana G, Butare L, Cregan PB, Blair MW, Kelly JD. 2014. Quantitative trait loci associated with drought tolerance in common bean. Crop Science. **54**, 923–938.

Muller B, Pantin F, Génard M, Turc O, Freixes S, Piques M, Gibon Y. 2011. Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. Journal of Experimental Botany **62**, 1715–1729.

Müller B deFaria, Sakamoto T, Silveira R, *et al.* 2014. Differentially expressed genes during flowering and grain filling in common bean (Phaseolus vulgaris) grown under drought stress conditions. Plant Molecular Biology Reporter **32**, 438–451.

Muñoz LC, Blair MW, Duque MC, Tohme J, Roca W. 2004. Introgression in common bean × tepary bean interspecific congruity-backcross lines as measured by AFLP markers. Crop Science. **44**, 637–645.

Muñoz-Perea C, Allen R, Westermann D, Wright J, Singh S. 2007. Water use efficiency among dry bean landraces and cultivars in drought-stressed and non-stressed environments. Euphytica **155**, 393–402.

Muñoz-Perea CG, Terán H, Allen RG, Wright JL, Westermann DT, Singh SP. 2006. Selection for drought resistance in dry bean landraces and cultivars. Crop Science. **46**, 2111–2120.

Murata N, Mohanty PS, Hayashi H, Papageorgiou GC. 1992. Glycinebetaine stabilizes the association of extrinsic proteins with the photosynthetic oxygen-evolving complex. FEBS Letters **296**, 187–189.

Nabhan G, Felger R. 1978. Teparies in Southwestern North America - biogeographical and ethnohistorical study of Phaseolus acutifolius. Economic Botany **32**, 2–19.

Navarro FM, Sass ME, Nienhuis J. 2009. Marker-facilitated selection for a major QTL associated with root rot resistance in snap bean (Phaseolus vulgaris L.). Crop Science **49**, 850–856.

Nayyar H. 2003. Accumulation of osmolytes and osmotic adjustment in water-stressed wheat (Triticum aestivum) and maize (Zea mays) as affected by calcium and its antagonists. Environmental and Experimental Botany **50**, 253–264.

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I. 2008. Nitric oxide, stomatal closure, and abiotic stress. Journal of Experimental Botany **59**, 165–176.

Nielsen DC, Nelson NO. 1998. Black bean sensitivity to water stress at various growth stages. Crop Science **38**, 422–427.

Nishizawa A, Yabuta Y, Shigeoka S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiology **147**, 1251–1263.

Obata T, Matthes A, Koszior S, Lehmann M, Araujo WL, Bock R, Sweetlove LJ, Fernie AR. 2011. Alteration of mitochondrial protein complexes in relation to metabolic regulation under short-term oxidative stress in Arabidopsis seedlings. Phytochemistry **72**, 1081–1091.

Okajima Y, Taneda H, Noguchi K, Terashima I. 2012. Optimum leaf size predicted by a novel leaf energy balance model incorporating dependencies of photosynthesis on light and temperature. Ecological Research **27**, 333–346.

Ogren WL. 1984. Photorespiration: pathways, regulation, and modification. Annual Review of Plant Physiology **35**, 415–442.

Park S-Y, Fung P, Nishimura N, *et al.* 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science **324**, 1068–1071.

Passioura JB. 1996. Drought and drought tolerance. Plant Growth Regulation **20**, 79–83.

Passioura JB. 2006. The perils of pot experiments. Functional Plant Biology **33**, 1075–1079.

Passioura JB. 2012. Phenotyping for drought tolerance in grain crops: when is it useful to breeders? Functional Plant BIology **39**, 851–859.

Pastenes C, Horton P. 1996. Effect of high temperature on photosynthesis in beans II. CO_2 assimilation and metabolite contents. Plant Physiology **112**, 1253–1260.

Pastenes C, Porter V, Baginsky C, Horton P, Gonzalez J. 2004. Paraheliotropism can protect water-stressed bean (Phaseolus vulgaris L.) plants against photoinhibition. Journal of Plant Physiology **161**, 1315–1323.

Peterhansel C, Krause K, Braun H-P, Espie GS, Fernie AR, Hanson DT, Keech O, Maurino VG, Mielewczik M, Sage RF. 2013. Engineering photorespiration: current state and future possibilities. Plant Biology **15**, 754–758.

Pimentel C, Ribeiro RV, Machado EC, dos Santos MG, de Oliveira RF. 2013. In vivo temperature limitations of photosynthesis in Phaseolus vulgaris L. Environmental and Experimental Botany **91**, 84–89.

Pinheiro C, Chaves MM. 2011. Photosynthesis and drought: can we make metabolic connections from available data? Journal of Experimental Botany **62**, 869–882.

Pinheiro H, DaMatta F, Chaves A, Loureiro M, Ducatti C. 2005. Drought tolerance is associated with rooting depth and stomatal control of water use in clones of Coffea canephora. Annals of Botany **96**, 101–108.

Pinto ME, Casati P, Hsu T-P, Ku MSB, Edwards GE. 1999. Effects of UV-B radiation on growth, photosynthesis, UV-B-absorbing compounds and NADP-malic enzyme in bean (Phaseolus vulgaris L.) grown under different nitrogen conditions. Journal of Photochemistry and Photobiology B: Biology **48**, 200–209.

Poorter H, Buehler J, van Dusschoten D, Climent J, Postma JA. 2012*a*. Pot size matters: a meta-analysis of the effects of rooting volume on plant growth. Functional Plant Biology **39**, 839–850.

Poorter H, Fiorani F, Stitt M, *et al.* 2012*b*. The art of growing plants for experimental purposes: a practical guide for the plant biologist Review. Functional Plant Biology **39**, 821–838.

Porch TG. 2006. Application of stress indices for heat tolerance screening of common bean. Journal of Agronomy & Crop Science **192**, 390–394.

Porch TG, Beaver JS, Brick MA. 2013. Registration of Tepary Germplasm with Multiple-Stress Tolerance, TARS-Tep 22 and TARS-Tep 32. Journal of Plant Registrations **7**, 358–364.

Porch TG, Jahn M. 2001. Effects of high-temperature stress on microsporogenesis in heat-sensitive and heat-tolerant genotypes of Phaseolus vulgaris. Plant, Cell & Environment **24**, 723–731.

Prasad PVV, Boote KJ, Allen LH, Thomas JMG. 2002. Effects of elevated temperature and carbon dioxide on seed-set and yield of kidney bean (Phaseolus vulgaris L.). Global Change Biology **8**, 710–721.

Qin X, Zeevaart JAD. 1999. The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. Proceedings of the National Academy of Sciences **96**, 15354–15361.

Rainey K, Griffiths P. 2005*a*. Differential response of common bean genotypes to high temperature. Journal of the American Society for Horticultural Science **130**, 18–23.

Rainey K, Griffiths P. 2005*b*. Evaluation of Phaseolus acutifolius A. Gray plant introductions under high temperatures in a controlled environment. Genetic Resources and Crop Evolution **52**, 117–120.

Ramalho JC, Zlatev ZS, Leitão AE, Pais IP, Fortunato AS, Lidon FC. 2014. Moderate water stress causes different stomatal and non-stomatal changes in the photosynthetic functioning of Phaseolus vulgaris L. genotypes. Plant Biology **16**, 133–146.

Rao I, Beebe S, Polania J, Ricaurte J, Cajiao C, Garcia R, Rivera M. 2013. Can tepary bean be a model for improvement of drought resistance in common bean? African Crop Science Journal **21**, 265–281.

Rasmussen S, Barah P, Suarez-Rodriguez MC, Bressendorff S, Friis P, Costantino P, Bones AM, Nielsen HB, Mundy J. 2013. Transcriptome responses to combinations of stresses in Arabidopsis. Plant Physiology **161**, 1783–1794.

Reich PB, Ellsworth DS, Walters MB, Vose JM, Gresham C, Volin JC, Bowman WD. 1999. Generality of leaf trait relationships: a test across six biomes. Ecology **80**, 1955–1969.

Richter JA, Erban A, Kopka J, Zörb C. 2015. Metabolic contribution to salt stress in two maize hybrids with contrasting resistance. Plant Science **233**, 107–115.

Rizhsky L, Liang H, Mittler R. 2002. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiology **130**, 1143–1151.

Rizhsky L, Liang H, Shuman J, Shulaev V, Davletova S, Mittler R. 2004. When defense pathways collide. The response of Arabidopsis to a combination of drought and heat stress. Plant Physiology **134**, 1683–1696.

Rolland F, Baena-Gonzalez E, Sheen J. 2006. Sugar sensing and signaling in plants: conserved and novel mechanisms. Annual Review of Plant Biology **57**, 675–709.

Rosales MA, Ocampo E, Rodríguez-Valentín R, Olvera-Carrillo Y, Acosta-Gallegos J, Covarrubias AA. 2012. Physiological analysis of common bean (Phaseolus vulgaris L.) cultivars uncovers characteristics related to terminal drought resistance. Plant Physiology and Biochemistry **56**, 24 – 34.

Rosales-Serna R, Kohashi-Shibata J, Acosta-Gallegos JA, Trejo-López C, Ortiz-Cereceres J, Kelly JD. 2004. Biomass distribution, maturity acceleration and yield in drought-stressed common bean cultivars. Field Crops Research **85**, 203–211.

Rut G, Rzepka A, Krupa J. 2010. Effect of hypoxia and post-hypoxia on the fluctuations in contents of malate and citrate, the activity of malic enzyme, and on the intensity of gas exchange in moss gametophores. Photosynthetica **48**, 79–86.

Sage RF. 2004. The evolution of C4 photosynthesis. New Phytologist **161**, 341–370.

Sage R, Kubien D. 2007. The temperature response of C-3 and C-4 photosynthesis. Plant Cell and Environment **30**, 1086–1106.

Sanders P, Markhart A. 1992. Interspecific grafts demonstrate root-system control of leaf water status in water-stressed phaseolus. Journal of Experimental Botany **43**, 1563–1567.

Schneider KA, Brothers ME, Kelly JD. 1997*a*. Marker-assisted selection to improve drought resistance in common bean. Crop Science **37**, 51–60.

Schneider KA, Rosales-Serna R, Ibarra-Perez F, Cazares-Enriquez B, Acosta-Gallegos JA, Ramirez-Vallejo P, Wassimi N, Kelly JD. 1997*b*. Improving common bean performance under drought stress. Crop Science **37**, 43–50.

Schramm F, Larkindale J, Kiehlmann E, Ganguli A, Englich G, Vierling E, Von Koskull-Döring P. 2008. A cascade of transcription factor DREB2A and heat stress transcription factor HsfA3 regulates the heat stress response of Arabidopsis. The Plant Journal **53**, 264–274.

Schmutz J, McClean PE, Mamidi S, *et al.* 2014. A reference genome for common bean and genome-wide analysis of dual domestications. Nat Genet **46**, 707–713.

Schwartz SH, Tan BC, Gage DA, Zeevaart JAD, McCarty DR. 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science **276**, 1872–1874.

Schymanski SJ, Or D, Zwieniecki M. 2013. Stomatal control and leaf thermal and hydraulic capacitances under rapid environmental fluctuations. PLoS ONE **8**, e54231.

Seiler C, Harshavardhan VT, Rajesh K, Reddy PS, Strickert M, Rolletschek H, Scholz U, Wobus U, Sreenivasulu N. 2011. ABA biosynthesis and degradation contributing to ABA homeostasis during barley seed development under control and terminal drought-stress conditions. Journal of Experimental Botany **62**, 2615–2632.

Seiler C, Harshavardhan VT, Reddy PS, *et al.* 2014. Abscisic acid flux alterations result in differential abscisic acid signaling responses and impact assimilation efficiency in barley under terminal drought stress. Plant Physiology **164**, 1677–1696.

Serraj R, Sinclair TR. 2002. Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant, Cell & Environment **25**, 333–341.

Seversike T, Sermons S, Sinclair T, Carter T Jr, Rufty T. 2014. Physiological properties of a drought-resistant wild soybean genotype: Transpiration control with soil drying and expression of root morphology. Plant and Soil **374**, 359–370.

Sharkey TD. 2005. Effects of moderate heat stress on photosynthesis: importance of thylakoid reactions, rubisco deactivation, reactive oxygen species, and thermotolerance provided by isoprene. Plant, Cell & Environment **28**, 269–277.

Sharkey TD. 2015. Commentary: what gas exchange data can tell us about photosynthesis. Plant, Cell & Environment.

Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. 2007. Fitting photosynthetic carbon dioxide response curves for C-3 leaves. Plant Cell and Environment **30**, 1035–1040.

Sharma DK, Andersen SB, Ottosen C-O, Rosenqvist E. 2012. Phenotyping of wheat cultivars for heat tolerance using chlorophyll a fluorescence. Functional Plant Biology **39**, 936–947.

Shulaev V, Cortes D, Miller G, Mittler R. 2008. Metabolomics for plant stress response. Physiologia Plantarum **132**, 199–208.

Sinclair TR, Zwieniecki MA, Holbrook NM. 2008. Low leaf hydraulic conductance associated with drought tolerance in soybean. Physiologia Plantarum **132**, 446–451.

Singh SP. 1995. Selection for water-stress tolerance in interracial populations of common bean. Crop Science **35**, 118–124.

Singh SP, Gepts P, Debouck DG. 1991. Races of Common Bean (Phaseolus vulgaris, Fabaceae). Economic Botany **45**, 379–396.

Singh SP, Muñoz CG. 1999. Resistance to common bacterial blight among Phaseolus species and common bean improvement. Crop Science. **39**, 80–89.

Singh J, Pandey P, James D, Chandrasekhar K, Achary VMM, Kaul T, Tripathy BC, Reddy MK. 2014. Enhancing C3 photosynthesis: an outlook on feasible interventions for crop improvement. Plant Biotechnology Journal **12**, 1217–1230.

Singh SP, Schwartz HF. 2010. Breeding common bean for resistance to diseases: a review. Crop Science **50**, 2199–2223.

Smartt J. 1981. Gene pools in Phaseolus and Vigna cultigens. Euphytica **30**, 445–449.

Smith AM, Stitt M. 2007. Coordination of carbon supply and plant growth. Plant Cell and Environment **30**, 1126–1149.

Somerville CR, Ogren WL. 1982. Genetic modification of photorespiration. Trends in Biochemical Sciences **7**, 171–174.

Souza RP, Machado EC, Silva JAB, Lagôa AMMA, Silveira JAG. 2004. Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (Vigna unguiculata) during water stress and recovery. Environmental and Experimental Botany **51**, 45–56.

Sreenivasulu N, Harshavardhan VT, Govind G, Seiler C, Kohli A. 2012. Contrapuntal role of ABA: Does it mediate stress tolerance or plant growth retardation under long-term drought stress? Gene **506**, 265–273.

Sreenivasulu N, Sopory SK, Kavi Kishor PB. 2007. Deciphering the regulatory mechanisms of abiotic stress tolerance in plants by genomic approaches. Gene **388**, 1–13.

Storey R, Ahmad N, Jones R. 1977. Taxonomic and ecological aspects of distribution of glycinebetaine and related compounds in plants. Oecologia **27**, 319–332.

Swindell W, Huebner M, Weber A. 2007. Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. BMC Genomics **8**, 125.

Terán H, Singh SP. 2002*a*. Comparison of sources and lines selected for drought resistance in common bean. Crop Science **42**, 64–70.

Terán H, Singh SP. 2002*b*. Selection for drought resistance in early generations of common bean populations. Canadian Journal of Plant Science **82**, 491–497.

Thompson AJ, Andrews J, Mulholland BJ, *et al.* 2007. Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. Plant Physiology **143**, 1905–1917.

Tian L, DellaPenna D, Zeevaart J. 2004. Effect of hydroxylated carotenoid deficiency on ABA accumulation in Arabidopsis. Physiologia Plantarum **122**, 314–320.

Timm S, Mielewczik M, Florian A, Frankenbach S, Dreissen A, Hocken N, Fernie AR, Walter A, Bauwe H. 2012. High-to-Low CO2 Acclimation reveals plasticity of the photorespiratory pathway and indicates regulatory links to cellular metabolism of Arabidopsis. PLoS ONE **7**, e42809.

Tolk JA, Howell TA, Miller FR. 2013. Yield component analysis of grain sorghum grown under water stress. Field Crops Research **145**, 44–51.

Udomprasert N, Li PH, Davis DW, Markhart AH. 1995. Effects of root temperatures on leaf gas exchange and growth at high air temperature in Phaseolus acutifolius and Phaseolus vulgaris. Crop Science. **35**, 490–495.

Urano K, Maruyama K, Ogata Y, *et al.* 2009. Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. Plant Journal **57**, 1065–1078.

Usadel B, Blaesing OE, Gibon Y, Retzlaff K, Hoehne M, Guenther M, Stitt M. 2008. Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. Plant Physiology **146**, 1834–1861.

Vadez V, Kholova J, Medina S, Kakkera A, Anderberg H. 2014. Transpiration efficiency: new insights into an old story. Journal of Experimental Botany **65**, 6141–6153.

Vassey T, Sharkey T. 1989. Mild water stress of Phaseolus vulgaris plants leads to reduced starch synthesis and extractable sucrose phosphate synthase activity. Plant Physiology **89**, 1066–1070.

Verbruggen N, Hermans C. 2008. Proline accumulation in plants: a review. Amino Acids **35**, 753–759.

Verslues PE, Juenger TE. 2011. Drought, metabolites, and Arabidopsis natural variation: a promising combination for understanding adaptation to water-limited environments. Current Opinion in Plant Biology **14**, 240–245.

Wahid A, Gelani S, Ashraf M, Foolad MR. 2007. Heat tolerance in plants: An overview. Environmental and Experimental Botany **61**, 199–223.

Wang W, Vinocur B, Shoseyov O, Altman A. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends in Plant Science **9**, 244–252.

Wanner LA, Junttila O. 1999. Cold-induced freezing tolerance in Arabidopsis. Plant Physiology **120**, 391–399.

Wentworth M, Murchie EH, Gray JE, Villegas D, Pastenes C, Pinto M, Horton P. 2006. Differential adaptation of two varieties of common bean to abiotic stress: II. Acclimation of photosynthesis. Journal of Experimental Botany **57**, 699–709.

Wenzel A, Frank T, Reichenberger G, Herz M, Engel K-H. 2015. Impact of induced drought stress on the metabolite profiles of barley grain. Metabolomics **11**, 454–467.

Westgate ME, Boyer JS. 1986. Reproduction at low and pollen water potentials in maize. Crop Science **26**, 951–956.

Wheeler MCG, Tronconi MA, Drincovich MF, Andreo CS, Flügge U-I, Maurino VG. 2005. A comprehensive analysis of the NADP-malic enzyme gene family of Arabidopsis. Plant Physiology **139**, 39–51.

White JW, Singh SP. 1991. Sources and inheritance of earliness in tropically adapted indeterminate common bean. Euphytica **55**, 15–19.

Wilkinson S, Davies W. 2002. ABA-based chemical signalling: the co-ordination of responses to stress in plants. Plant Cell and Environment **25**, 195–210.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. An 'electronic fluorescent pictograph' browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2**, e718.

Witt S, Galicia L, Lisec J, Cairns J, Tiessen A, Luis Araus J, Palacios-Rojas N, Fernie AR. 2012. Metabolic and phenotypic responses of greenhouse-grown maize hybrids to experimentally controlled drought stress. Molecular Plant **5**, 401–417.

Wong SC, Cowan IR, Farquhar GD. 1979. Stomatal conductance correlates with photosynthetic capacity. Nature **282**, 424–426.

Wright IJ, Reich PB, Cornelissen JHC, *et al.* 2005. Assessing the generality of global leaf trait relationships. New Phytologist **166**, 485–496.

Xiao X, Yang F, Zhang S, Korpelainen H, Li C. 2009. Physiological and proteomic responses of two contrasting Populus cathayana populations to drought stress. Physiologia Plantarum **136**, 150–168.

Xing W, Rajashekar C. 1999. Alleviation of water stress in beans by exogenous glycine betaine. Plant Science **148**, 185–192.

Xing W, Rajashekar C. 2001. Glycine betaine involvement in freezing tolerance and water stress in Arabidopsis thaliana. Environmental and Experimental Botany **46**, 21–28.

Yang SH, Zeevaart JAD. 2006. Expression of ABA 8`-hydroxylases in relation to leaf water relations and seed development in bean. Plant Journal **47**, 675–686.

Yang J, Zhang J. 2006. Grain filling of cereals under soil drying. New Phytologist **169**, 223–236.

Yin Y, Adachi Y, Ye W, Hayashi M, Nakamura Y, Kinoshita T, Mori IC, Murata Y. 2013. Difference in abscisic acid perception mechanisms between closure induction and opening inhibition of stomata. Plant Physiology **163**, 600–610.

Yurekli F, Porgali Z, Turkan I. 2004. Variations in abscisic acid, indole-3-acetic acid, gibberellic acid and zeatin concentrations in two bean species subjected to salt stress. Acta Biologica Cracoviensia Series Botanica **46**, 201–212.

Zadražnik T, Hollung K, Egge-Jacobsen W, Meglič V, Šuštar-Vozlič J. 2013. Differential proteomic analysis of drought stress response in leaves of common bean (Phaseolus vulgaris L.). Journal of Proteomics **78**, 254–272.

Zelitch I, Schultes NP, Peterson RB, Brown P, Brutnell TP. 2009. High glycolate oxidase activity is required for survival of maize in normal air. Plant Physiology **149**, 195–204.

Zhang J, Nguyen HT, Blum A. 1999. Genetic analysis of osmotic adjustment in crop plants. Journal of Experimental Botany **50**, 291–302.

Zinselmeier SA, Lauer MJ, Boyer JS. 1995. Reversing drought-induced losses in grain yield: sucrose maintains embryo growth in maize. Crop Science **35**, 1390–1400.

Zlatev ZS, Lidon FC, Ramalho JC, Yordanov IT. 2006. Comparison of resistance to drought of three bean cultivars. Biologia Plantarum **50**, 389–394.

Zwack PJ, Rashotte AM. 2015. Interactions between cytokinin signalling and abiotic stress responses. Journal of Experimental Botany **66**, 4863–4871.