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Identification of Physiological and Morphological Traits Governing High Water Use Efficiency in Alfalfa

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IDENTIFICATION OF PHYSIOLOGICAL AND MORPHOLOGICAL TRAITS

GOVERNING HIGH WATER USE EFFICIENCY IN ALFALFA

BY

KRISHNA GHIMIRE

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Biology

South Dakota State University

2017

IDENTIFICATION OF PHYSIOLOGICAL AND MORPHOLOGICAL TRAITS

GOVERNING HIGH WATER USE EFFICIENCY IN ALFALFA

KRISHNA GHIMIRE

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Science degree and is acceptable for meeting the thesis for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

This thesis is a great achievement of my academic career at South Dakota State University. It is my great pleasure to acknowledge all those people who made this thesis possible with their continuous help and support.

I would like to express my deep gratitude to my advisor Dr. Yajun Wu for his support, encouragement, valuable advice and feedback. He helped me strengthen my work. I would like to thank my committee members, Dr. Donald Auger and Dr. Yang Yen, for their suggestions and feedback for my work.

I would like to acknowledge Department of Biology and Microbiology for providing me opportunity to enroll in SDSU as a graduate student. The department has provided me teaching assistantship that not only supported me financially but also helped me gain valuable teaching skills.

Finally, I would also like to thank my wife and parents for their love and support.

Krishna Ghimire

South Dakota State University

Brookings, SD

March 2017

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ABSTRACT

IDENTIFICATION OF PHYSIOLOGICAL AND MORPHOLOGICAL TRAITS

GOVERNING HIGH WATER USE EFFICIENCY IN ALFALFA

KRISHNA GHIMIRE

2017

Alfalfa is an important forage crop worldwide. Being deep-rooted, N_2-fixing and high yielding, alfalfa has great economic, ecological and nutritional benefits. While alfalfa is a high yielding crop, its high productivity depends on irrigation water in many areas and consumes the greatest amount of water among all the major crops. With a growing demand for water resources due to an increase in human population and industrial water use, plus frequent drought due to climate change, irrigation water has become increasingly scarce and expensive. To sustain high production of alfalfa with limited water resource, alfalfa cultivars with improved water use efficiency (WUE) is urgently needed. As a first step, we started screening alfalfa germplasms for difference in WUE and identified an alfalfa collection, River side (RS), with a greater WUE under drought compared to other ten alfalfa collections. RS is a naturalized alfalfa collected from the Grand River National Grassland in South Dakota. The objective of this study was to identify physiological and morphological traits that may contribute toward higher WUE in RS. Plants were subjected to two water regimes, by supplying either 100% (well-watered) or 50% (water-stressed) of their transpirational water needs. RS showed the smallest stomatal conductance and used the least amount of water under drought compared to other two alfalfa collections, suggesting that a greater WUE in RS is associated with a reduced transcriptional water

loss. We found that RS developed smaller but more numerous stomata under drought that might facilitate a more rapid stomatal closure when water is limited but enhance water and nutrient uptake when water is sufficient. RS has also exhibited different changes on two sides of the leaf that may contribute to the regulation of water loss. The abaxial surface developed a greater number of leaf hairs that can potentially increase the boundary layer resistance for transpiration. The adaxial surface developed the stomata with a greater sensitivity to ABA. By examining the leaf epidermal cell size, it is clear that RS showed the greatest reduction in cell size, resulting in a great increase in cell density. The change in cell density may explain an increased stomatal and leaf hair density observed. Our study provided a great insight into the factors that may contribute to a high WUE in alfalfa. We hope that the knowledge developed in this study and in the future study will build a foundation for developing alfalfa with improved WUE.

CHAPTER 1. LITERATURE REVIEW

1. ALFALFA AND ITS INTRODUCTION

Alfalfa (*Medicago sativa* L.), known as queen of forage, is the most important and the oldest forage crop in the world (Michaud et al. 1988). Alfalfa is a perennial leguminous crop which is cultivated worldwide for hay, pasture and silage because of its highly nutritious value, broad adaptability, and significance in other applications. The center of origin of alfalfa is Asia Minor, Transcaucasia, Turkmenistan and Iran (Quiros and Bauchan 1988). Alfalfa was introduced to the US from different sources. It was introduced from British Isle to Georgia in 1836 and to Utah in 1850, from Chile and Mexico to California in 1850 and 1851, from Germany to Minnesota in 1857. However, the introduction to California from Chile in 1850 caused its eventual spread across USA (Brough et al. 1973).

2. IMPORTANCE OF ALFALFA

2.1. Economic importance

Alfalfa is a highly valued crop because of its close link with the dairy and beef industries (Sumner and Rosen-Molina 2011). Alfalfa production and productivity has increased during the past several years in the US. The total area harvested was 16.75, 17.67, 18.44 million acres in 2012, 2013, and 2014, respectively (NASS 2015), with a productivity of 3.01, 3.24, and 3.33 tons per acre in 2012, 2013, and 2014 respectively. In 2014, alfalfa hay produced in the US valued 10.7 billion dollars (NASS 2015). Since alfalfa is used in animal feed for dairy cows, horses,

sheep, turkey, chicken and other farm animals, the total value of alfalfa is worth 145 billion dollars (Edminster et al. 2001)

2.2. Ecological importance

Alfalfa is ecologically important because of its deep root system and perennial nature. It limits soil erosion and improves soil texture (Li and Brummer 2012). Being a legume, alfalfa has a symbiotic relationship with N_2 fixing bacterium *Sinorhizobium meliloti* that fixes atmospheric nitrogen and improves the fertility of soil for subsequent crops. Thus, less nitrogenous fertilizer is needed for subsequent crops during crop rotation. This rotation of alfalfa with other crops not only reduces the expense on fertilizer application but also minimizes the runoff of excessive fertilizer that negatively impacts the environment (Acharya et al. 2013). Alfalfa is also used for phytoremediation, preventing surface water pollution (Vadas et al. 2008). Alfalfa also provides habitat to different wildlife species (Putnam et al. 2001; Shebl et al. 2008; Putnam 2004).

2.3. Industrial importance

In addition to its high value in dairy and ranch industry, alfalfa shows its potential in other industrial applications. For example, alfalfa has recently been proposed a great source for cellulosic biomass for biofuel production. Alfalfa is one of the highest biomass producers among all the major crops. Since alfalfa production needs little nitrogen fertilizer, it is considered a low-input cellulosic biomass crop. Alfalfa has about 50% of polysaccharides in dry weight that can be used for producing biofuel and chemical products (del Pozo et al. 2017). Great effort has been made to improve alfalfa biomass production for biofuel production purpose

such as using unique germplasm (Lamb et al. 2007). The stem cell wall alternation such as reducing lignin content is another approach used to change cell wall quality and to improve the efficiency of cellulosic ethanol production (Lamb et al. 2007; Gronwald 2009).

2.4. Nutritional importance

Forage and legume crop is the foundation of dairy and meat production for centuries (Russelle et al. 2001). Alfalfa has a high content of digestible energy and protein which makes it an extremely valuable feed (Yi et al. 2013). When included in livestock ration it can reduce or eliminate the need of supplemental protein feed. Alfalfa is also high in calcium, magnesium and phosphorous (Garry Lacefield 2009).

3. ALFALFA PRODUCTION AND ITS CONSTRAINTS

Although alfalfa is a high-yield crop and is grown worldwide for different purposes, there are many constraints that limit its production and productivity.

3.1. Alfalfa production

North America, Europe and South America are leading alfalfa producers. In term of acreage leading alfalfa producing countries are the USA, Argentina, Canada, Russia, Italy and China. In USA alfalfa is the fourth largest crop in terms of area cultivated after corn, wheat and soybean (Yuegao and Cash 2009). The primary global center for alfalfa cultivation is North America which has more than half the total worldwide acreage (Bagavathiannan and Van Acker 2009). While the production of alfalfa increased in the US in the past, global alfalfa production

declined slightly since the start of the 21st century due to fluctuations in economics of grain crops, animal husbandry and expansion of soybean and corn for bioenergy (Yuegao and Cash 2009). Alfalfa production in Europe has also declined since 2000. Due to a rapid expansion of the dairy industry, demand for alfalfa is high in China. Although China is currently an importer of alfalfa, it has the huge potential to become a sustainable production system. The USA exports a large quantity of alfalfa hay to China, Japan, Korea, Taiwan, and the UAE. Alfalfa production in North America and Europe is expected to increase in response to an increases in alfalfa price in international markets (Yuegao and Cash 2009)

3.2. Constraints of alfalfa production

In general, alfalfa is relatively tolerant to different abiotic stresses like drought, heat and cold, and it thus has been grown in many regions of the world (Leach and Clements 1984). However, its potential for high yield is limited by these abiotic stresses. In this study, we mainly focused on the impact of water availability on alfalfa production and responses of plants to water deficit.

3.2.1. Irrigation water availability

Agriculture uses 70% of overall withdrawal of global water resources (Morison et al. 2008), and low water availability is the primary factor limiting plant growth and yield worldwide (Chaves et al. 2003). Alfalfa consumes a great amount of water and its yield is limited by irrigation rather than any other management factors. Evapotranspiration (total water use) for alfalfa generally varies from 0.1 to 0.35 inches per day (Irmak et al. 2007). Alfalfa is ranked number one for irrigation water use among the major crops when

irrigation is used in many regions in order to achieve high yields (Irmak et al. 2007; Putnam 2012)

There is, however, an increasing competition for water use between agricultural and other purposes due to limited water availability. Increasing water demand for municipal and urban use and decreasing ground water level and precipitation have caused the reduced quantities of irrigation water available in agriculture (Lindenmayer et al. 2008). Climate change may exacerbate the present water shortage and increase the pressure on irrigation water use in agriculture (Alley and Berntsen 2007). Many countries have made laws to regulate water use, and farmers are under legislative restriction for irrigation water use (e.g. the 2003 Water Act in the UK). These laws are made to secure safe and adequate water for domestic use (Morison et al. 2008). The UAE government decided to stop alfalfa production in the kingdom in 2008 due to their increasingly scarce water resources. Saudi Arabia purchased a large acreage of land in California to produce alfalfa to preserve their own scare water resource and transport alfalfa hay from California to the Middle East (Jeff Denials, 2015).

Alfalfa is grown on 12% of irrigated land in the USA (NASS, 2007) and the cost for irrigation is high and is increasing. The cost is even higher during dry seasons as it produced the lowest yield per unit water used. This is because high irrigation at hot and dry conditions is wasteful through high evapotranspiration (Morison et al. 2008). Thus, reduced irrigation on alfalfa could save great quantities of water without negative impact on overall

financial return for alfalfa growers in some drought conditions (Parry et al. 2005).

3.2.2. Climate change and drought

Sixty seven percent of crop loss in the USA for the past 50 years is due to drought. The 2012 drought in the USA was the worst in the last 60 years. Intergovernmental Panel on Climate Change has predicted that there will be a decrease in precipitation and rising evapotranspiration (Alley and Berntsen 2007). As a result, more frequent water shortages are expected due to the climate change and increasing competition of water use between agriculture, industrial areas and urban area (Field 2012; von Mogel 2013). The problem of drought for legume production will be worsened as it is projected that rapidly increasing water stressed areas in the world will expand from 28 to 30 countries in 2000 to 50 countries with 3 billion people by 2030 (Postel 2000). Alfalfa production is also facing the challenge of frequent drought. Identification and generation of alfalfa cultivars that remain highly productive with less irrigation is urgently needed.

4. WATER USE EFFICIENCY (WUE)

Water use efficiency (WUE) is defined as a ratio of biomass production to water use. WUE is considered an important trait that determines yield during drought. A higher WUE is derived mainly due to plant traits that reduce transpiration and crop water use (Blum 2009). Reducing transpiration would decrease water loss but often decrease biomass production as well due to a lower photosynthesis. Thus, one strategy is to

develop a crop with high WUE with little effect on biomass yield. Alternatively, breeding alfalfa for increased soil moisture capture can be an important target to improve yield under drought.

When plants are under moderate water deficit stress, stomata are closed by sensing physical and chemical signals such as hydraulic pressure and abscisic acid (ABA). This decline in stomatal conductance reduced both photosynthetic rate and transpiration. Transpiration is the major cause of water loss in photosynthetic plants. An increase in WUE often occurs because inhibition of transpiration is more than photosynthesis (Xu et al. 2010). WUE has a genetic basis, and breeding for high WUE has become a main objective for the breeding program of many crops (Condon et al. 2004). Studies have demonstrated that genetic variation in WUE is mainly due to a variation in stomatal conductance but not net assimilation (Blum 2009). Thus, the key to improve WUE in plants is to control stomatal conductance.

5. IMPACT OF DROUGHT ON PLANT GROWTH AND PLANT RESPONSES TO DROUGHT

Alfalfa is a relatively "drought-tolerant" as compared to other crops due to its deep root system which allows to extract water deeper in the soil (Tang et al. 2013; Moran et al. 1994). Plant growth and survival depend on water availability. When water availability changes, plants modulate their physiological and developmental processes and keep balances between water use and water uptake (Hamanishi et al. 2012)

5.1. Plant growth

The abiotic and biotic stress has caused the evolution of modern plants from the primitive living organisms. Among many abiotic stress which shaped the form of plant evolution, water stress or drought is the most important one (Zhu 2002). Since water is a key substrate for photosynthesis and sustains turgor pressure that drives cell expansion, photosynthesis and cell elongation are primarily affected by drought. The sensitivity and response time to drought differ among species, and different mechanisms have been adopted by plants to respond to drought stress (Aasamaa and Sõber 2011). Two of the important mechanisms in response to drought are to minimize water use by mainly limiting leaf and shoot growth and to reduce water loss by mainly reducing transpiration.

5.1.1. Leaf growth, development and morphology

Plants respond to water deficit condition by reducing leaf area, which reduces water use needed for cell expansion. At the same time, other morphological and physiological changes occur in plants that allows plants to reduce transpiration and retain water thus increasing WUE (Xu and Zhou 2005).

5.1.1.1.   Leaf area and thickness

During drought, leaf area expansion is suppressed earlier than photosynthesis (Tardieu et al. 1999). Reduced leaf area, leaf area index (defined as the ratio of total one sided area of leaf tissue to ground surface area) and reduced plant size are important mechanisms to reduce water use (and loss) and minimize stress during drought (Mitchell et al. 1998). Specific leaf area is also changed during drought, which is an indicator

of leaf thickness. These modified leaf morphologies allows plants to increase leaf protein density thus consequently increases photosynthesis per unit volume of leaf (Marcelis et al. 1998)

5.1.1.2.   Leaf hair density

Leaf hairs play important physiological and ecological roles in plants. Leaf hairs help in defense against herbivores, and it also affects gas exchange and leaf temperature. The trichrome (leaf hair) prevents the absorption of short wave length of light and makes leaf cooler (Baldocchi et al. 1983). Leaf hairs also keep water droplets off the leaf and stomata thus facilitating gas exchange (Brewer and Smith 1995). Leaf hairs influence transpiration also. The transpiration is directly proportional to water vapor concentration between ambient air and sub stomatal cavity. Thus, thickening of boundary layer by leaf hairs increases the resistance to water vapor diffusion and decreases the transpirational water loss (Wuenscher 1970). Effect of drought stress on leaf hair density was observed in *Piriqueta caroliniana*, which produced less leaf hairs in wetter climate and more leaf hairs in drier climate (Picotte et al. 2009). Leaf hair density has shown to increase WUE in drought condition (Picotte et al. 2007).

5.1.1.3.   Stomatal development

Stomata are the pores on the leaf epidermis that consist of two specialized guard cells that surround the central aperture. Stomata control the gaseous exchange between leaf and atmosphere. Development of stomata is said to be the most important development in plant evolution (Brodribb and McAdam 2011). Stomatal evolution is a result of response to selection pressure to optimize the ratio of CO_2 uptake to water loss during photosynthesis (Raven 2002). The short term response of plants to water deficit is the decrease in stomatal aperture to reduce water loss, which often negatively impacts photosynthesis at the same time (Chaves et al. 2003). During persistent water stress conditions, plants can control the stomatal number by modulating stomatal development during new leaf development (Chaves et al. 2003). The stomatal effects are the most important factor limiting photosynthesis during moderate drought while biochemical limitations in photosynthesis are more important during severe drought (Grassi and Magnani 2005).

5.2. Signaling molecules and action under drought

Multiple signaling molecules or systems have been reported to be important for drought response in plants. These molecules are produced in roots under drought stress and then transported to shoots via xylem to reduce leaf growth and stomatal aperture thus transpirational water loss. Among those stress signaling molecules, abscisic acid (ABA) is known as a key stress hormone that is involved in root-

shoot communication (Chaves et al. 2003). Water deficit activates biosynthesis, accumulation and redistribution of ABA which reduces the stomatal aperture in leaf and thus minimizing transpirational water loss (Hirayama and Shinozaki 2007). Besides ABA, other phytohormones like jasmonic acid, cytokinin, ethylene or brassinosteroids are also involved in stomatal response to stress (Daszkowska-Golec and Szarejko 2013). Other signal molecules, such as nitric oxide (NO), hydrogen peroxide (H_2O_2), reactive oxygen species (ROS) are also involved in drought response and tolerance. A major role of these molecules in stress response is to act in the pathways of ABA to regulate stomatal closure under drought (Lu et al. 2009; Bright et al. 2006; Cruz de Carvalho 2008).

5.3. Regulation of stomatal closure and opening under drought

Stomata control the gaseous exchange between leaf and atmosphere thus regulating CO_2 uptake and transpiration, thus effecting the plant productivity and WUE (Lawson and Blatt 2014). Ninety percent of water loss from plants is through stomatal pores. The balance between CO_2 uptake and transpiration depends on stomatal response to environmental conditions and internal cues. There exist significant differences in sensitivity and responsiveness of stomata to different environmental conditions such as light and humidity and internal cues such as hormones and CO_2 level (Lawson et al. 2012). These external and internal factors control the stomatal closure and opening by mostly regulating the turgor pressure inside the guard cells.

The stomatal opening occurs when a guard cell becomes turgid because of osmotic uptake of water into the guard cells and stomata close when guard cells lose water

and become flaccid. Change in guard cell turgor is controlled largely by influx and efflux of K^+ ion in exchange with Cl^- or malate ion across plasma and tonoplast membrane (Hetherington 2001). ABA controls activity of three major channels, inward and outward K^+ channel and anion channel at guard cell plasma membrane to achieve net efflux of osmotic solutes (thus water) to achieve stomata closure (Armstrong et al. 1995). Extensive studies have revealed a sophisticated signaling network in guard cells involving stress hormone ABA (Fig.1) (Daszkowska-Golec and Szarejko 2013). ABA regulates many downstream components such as protein kinase, phosphatase, increase in cytosolic pH, increase of free Ca^+ in guard cells, which in turn regulates ion channel activity and turgor pressure (Hetherington 2001). Hydrogen peroxide (H_2O_2) (Pei et al. 2000) and nitrous oxide (NO) (Neill et al. 2002) has been identified as key signaling molecules in ABA-mediated stomatal closure. ABA induces H_2O_2 production, and H_2O_2 then activates the Ca^{2+} channels to increase cytosolic calcium in guard cells (Pei et al. 2000).

A difference in ABA metabolism and signaling was observed in *Vitis* genotypes that showed different levels of drought adaptation (Rossdeutsch et al. 2016). While a stress-induced accumulation of ABA is a key to the stomatal closure in many plants, many studies also showed that guard cells in some plants become more sensitive or responsive to ABA. Differences in stomatal response to ABA were observed among *Vitis* and wheat genotypes (Hopper et al. 2014; Chen et al. 2013). A correlation between ABA sensitivity and drought tolerance has been observed in synthetic wheat (Kurahashi et al. 2009).

6. OBJECTIVE OF THE STUDY

Alfalfa has high WUE in terms of biomass production vs irrigation water input and it is one of the crop with highest WUE (Putnam 2012; Asseng and Hsiao 2000). Although alfalfa has high WUE, it is one of the crop that consumes the greatest amount of irrigation water among all the major crops (Schneekloth and Andales 2009; Hanson et al. 2008). The high water use in alfalfa is due to its longer growing season, dense canopy cover and high biomass yield. Irrigated alfalfa has a high evapotranspiration demand, which probably is linked to its morphology. Alfalfa has a higher stomata density compared to most of the plants. In addition, alfalfa leaves have stomata on both adaxial and abaxial surfaces (amphistomatous). While it is not uncommon in many plants that stomata can be found on both surfaces, plants usually have more stomata on the lower or abaixal surface (Tari 2003). Leaves of alfalfa, however, have more stomata on adaxial surface than abaxial surface (Cole and Dobrenz 1970). Amphistomaty is related with high conductance to CO_2 and is advantageous to plants that benefit from high maximum conductance and adaptive for plants with high photosynthetic capacity, living on high sunlight areas and able to utilize water quickly when available (Mott 1982). Stomata distribution on abaxial surface would reduce transpiration (Rushin and Anderson 1981). Alfalfa also has deep roots that can extract water from deeper soil when surface water is limited. This allows them to avoid mild drought stress. These characteristics suggest that alfalfa is a high-yielding but high water consuming plant that may not have been subjected to selection for WUE, since it is able to compete for water with other plants through its advanced root system. Despite its

high biomass production and high WUE in using irrigation water, its large consumption of irrigation water may not be sustainable in the environment that water resources become increasingly scarce. Thus, it is urgent to develop alfalfa with improved WUE. As a first step, we examined 11 alfalfa collections for variation in WUE under drought. Water supply was adjusted based on their transpirational water loss and water supply for individual plants was maintained at 100%, 75%, 50% and 25% of transpirational water loss. We identified one alfalfa accession, River side, that showed significantly higher WUE under drought conditions compared to other accessions (Anower et al. 2015). In this present study, we examined morphological and physiological traits that may contribute towards the high WUE in River side.
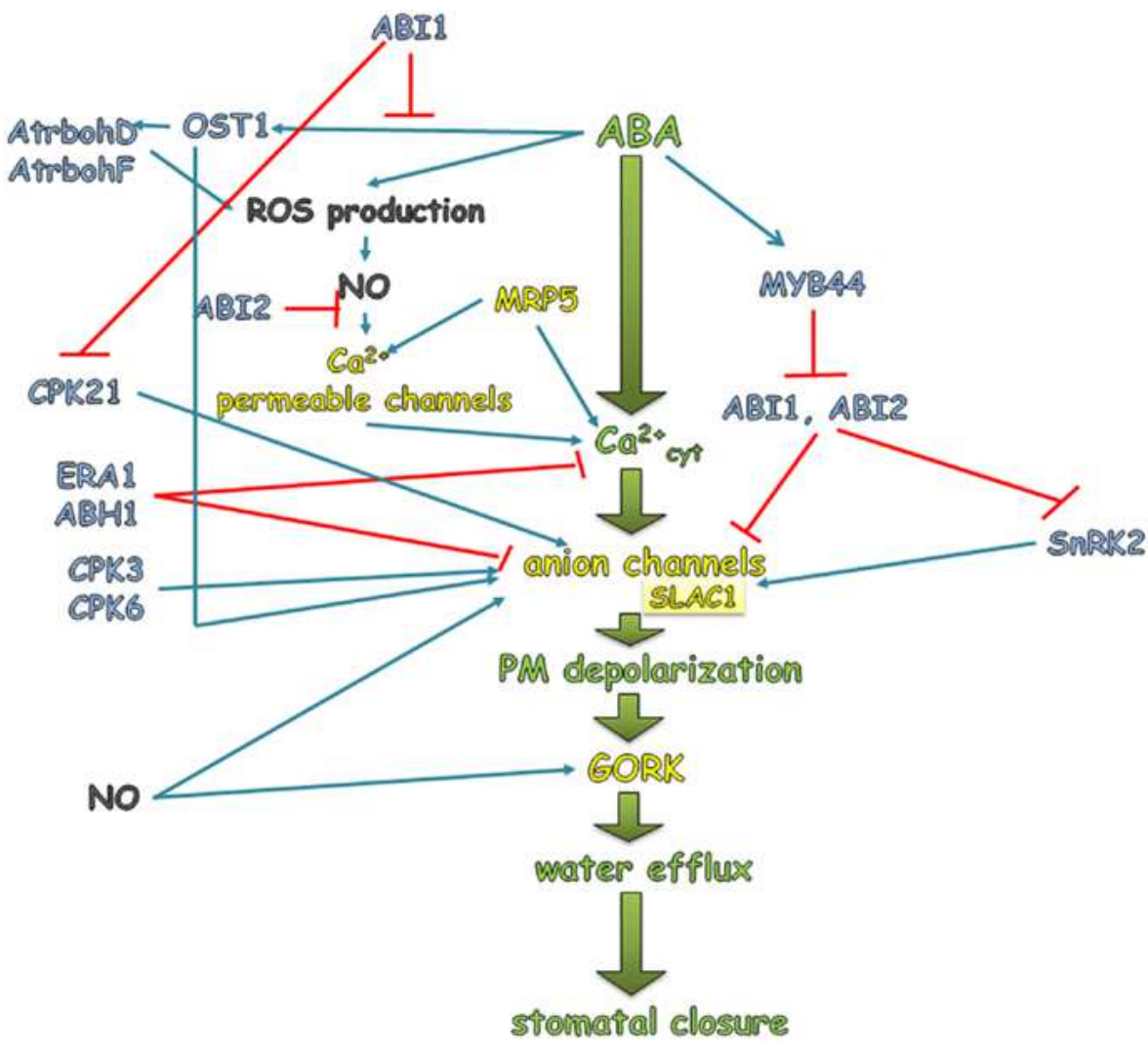
Figure 1. ABA induced stomata closure (Daszkowska-Golec and Szarejko 2013)

Chapter 2. Identification of physiological and morphological traits governing high WUE in alfalfa

INTRODUCTION

Alfalfa is an important leguminous forage crop and cultivated worldwide. It is used as green forage, hay and silage. The alfalfa production is negatively affected by various biotic and abiotic factors. The most significant abiotic stress limiting crop production is drought. Plants have developed various physiological and morphological traits to adapt to drought conditions (Blum 1996) and are shaped by evolution to reduce water use during drought (Blum 2009). Irrigation is used to alleviate yield reduction due to drought. Agriculture use 70% of global water withdrawn from surface water and underground water (Wisser et al. 2008). The increasing incidence of drought has put more pressure on irrigation as there will be more water demand for domestic and industrial use because of growing human population. Because of insufficient irrigation water, agriculture will shift from the goal of more production per unit area toward more production per unit water used (Fereres and Soriano 2007). Thus, improving the water use efficiency of crop will be an important target of crop breeding programs.

Water use efficiency (WUE) is defined as the biomass produced per unit water used and is a critical factor determining yield under drought (Saranga et al. 1999). Drought affects stomatal conductance, which influences overall photosynthesis and transpiration. In general, there is a strong positive correlation between photosynthesis and stomata conductance (Jones 1998; Sperry et al. 2002). Drought induced stomatal closure significantly reduces stomatal conductance and transpiration but also decreases photosynthesis (Miyashita et al. 2005), but overall WUE of plants increases because the

decrease in stomatal conductance/water loss is more than in photosynthesis (Edwards et al. 2012). Thus, the variation of WUE of crops under drought is mostly determined by total water use rather than net biomass accumulation (Blum 2005). The plants exhibit high WUE when stomatal conductance is lower than the potential maximum. Therefore, WUE can be improved without much negative impact on yield. Increasing WUE is critical for the regions with increasing drought and diminishing ground water level (Franks et al. 2015). In addition, plants with higher WUE are expected to survive more arid conditions than plants with lower WUE (Franco et al. 2005; Ares et al. 2000)

The stomatal conductance determines the rate of gas exchange (movement of carbon dioxide for photosynthesis into leaf and transpiration of water vapor out of leaf) through leaf's stomata. Thus, stomatal conductance is the function of number of stomata and its distribution in abaxial and adaxial surface, size of stomata, and degree of opening of stomata. During drought plants increase WUE by reducing the stomatal aperture as a short-term response. Under prolonged drought, some plants increase WUE by developing leaves with altered stomatal density and size as well as with reduced stomatal conductance (Doheny-Adams et al. 2012). In addition to stomatal density and size, the distribution of stomata and leaf epidermal structure such as wax deposition and leaf hairs may also contribute to transpiration thus WUE. Studies showed that stomatal density increases (Yang and Wang 2000; Zhang et al. 2005) while the size decreases under drought (Xu and Zhou 2008; Martínez et al. 2007). The decrease in size with respect of increase in density help plants regulate water use more efficiently as there are suggestion that small stomata can be easily closed and opened (Raven 2014). Smaller stomata shows higher water use efficiency (Aasamaa et al. 2001; Hetherington and Woodward 2003). Many plants have

dense trichomes or leaf hairs on their leaf surface. They may serve as a barrier against external herbivores, protection against UV-B radiation, high temperature and excessive water loss (Werker 2000). Trichomes reduce transpiration by increasing boundary layer (Skelton et al. 2012) and significantly contribute towards the overall gas (including water vapor) exchange resistance in some plants (Ripley et al. 1999).

Stomata are the gateways that help gaseous exchange between plant and environment. Two guard cells that surround the stomatal pore help to optimize the tradeoff between water vapor loss and carbon gain (Kim et al. 2010). The regulation of stomatal aperture size is critical, and it is dynamic and reversible process that allows regulation of loss of water and influx of CO_2 tuned with environmental signals and internal cues like light, CO_2 and plant hormone abscisic acid (Schroeder et al. 2001). Abscisic acid (ABA), also known as a stress hormone, accumulates under drought. ABA produced in roots moves to shoot through xylem acting as a long distance signal for water stress response and reduces stomata aperture as well as leaf growth (Zhang and Davies 1989) (Davies and Zhang 1991). ABA triggers various downstream responses and helps plants resist drought stress (Shinozaki and Yamaguchi-Shinozaki 1997). One of the major functions of ABA is regulating the water status of plants by controlling the guard cells therefore stomatal closure under drought. Extensive studies have revealed a complex signaling cascade triggered by ABA to induce stomatal closure under drought (Fig. 1).

Alfalfa is a high biomass-producing crop. However, its production in many areas consumes the greatest amount of agricultural irrigation water (Hanson et al. 2008; Schneekloth and Andales 2009). Improving WUE in alfalfa is one of the major strategies to reduce irrigation use. Our lab recently identified an alfalfa germplasm, River side (RS), that is naturally

adapted to the Grand River National Grassland region in South Dakota and has exhibited higher WUE over several other germplasms under drought condition (Anower et al. 2015). The aim of this study is to identify the morphological and physiological traits that may contribute to a higher WUE in RS.

MATERIALS AND METHODS

Plant materials

Three alfalfa (*Medicago sativa* L.) collections, River side (RS), a collection that is naturalized to the Grand River National Grassland in South Dakota (SD), Foster ranch (FR) collected from Thunder Butte Creek (45°N, 101°W), North of Faith, SD, and Alfagraze (AF), a commercial variety, were used in this study. The plants for the experiment were propagated through cuttings and rooting in cone containers. Each cone container was filled with 40 grams potting mixture (Sunshine Mix #3, Sun Gro Horticulture Canada Ltd., Seba Beach, AB, Canada). The bottom of cone container was blocked with a paper towel to hold the soil while the excess water could drip out of the container. For cutting, healthy shoots from mother plants were selected. The apical 2-3 internodes and the basal internodes were cut and discarded. The middle 2-3 internodes were retained. A slanting cut was made at the basal end. The slanting end was quickly dipped into rooting hormone (IBA) (Hormex rooting power no. 16, Brooker Chemical, Chastsworth, CA, USA) and planted into the cone container containing water saturated potting mix. The cuttings were watered daily. Once the new branch emerged from the cuttings the healthy plants with similar size were selected and used for the experiments.

Drought treatment

Two irrigation treatments were conducted based on transpirational water loss of individual plants. The selected plants were fully watered at the day of onset of experiment, and the containers along with plant were weighed. The top surface of each cone container was covered with aluminum foil to eliminate evaporation form soil surface. Each container was weighed in 3-day intervals to determine the transpirational water loss. The plants were either irrigated with the amount of water to fully compensate for the water loss due to transpiration (W100 or control) or to only compensate for 50% of transpirational water loss (W50 or drought treatment). The water was delivered slowly to each cone container using a plastic pipette to ensure all the water was captured by the potting mix. The treatments were continued for four weeks. A mild water stress was used in this study so that the features examined in this study can be associated with transpirational regulation.

Stomatal conductance measurement

The stomatal conductance was measured using a portable leaf porometer (SC-1, Decagon, city?, State?). The leaf porometer was first calibrated by following the protocol provided in the manual (https://www.decagon.com/en/support/videos/sc-1-leaf-porometer-calibration). The mature leaves were selected for measurement. The stomatal conductance was recorded on both abaxial and adaxial surfaces of the leaf. The conductance measurements were done from 10 am to noon.

Stomata number

Leaf imprints technique was used to count the stomata number on both surfaces of the leaf. A thin layer of clear nail polish was applied on leaf surface. Once the nail polish was dry,

the film on the leaf surface was peeled off using a clear tape. The nail polish film with imprints of leaf was mounted on a microscope slide and observed under a light microscope (ATC 2000, Leica). A stage micrometer was used to determine the area of view to calculate the stomatal density. Stomata were counted at random spots on each surface of the leaf. At least three leaves were selected for measuring stomata number from each of the three plants in each treatment.

Leaf hair density

The leaf hair density was measured on the abaxial leaf surface. The leaves were harvested and directly observed under a dissecting microscope. Total number of leaf hairs visible within the field of view was counted. The field of view was determined using a scale to be 4.5 mm in diameter, i.e. 15.9 mm^2.

Stomatal sensitivity to abscisic acid

The youngest mature leaves were selected for measurement. The harvested leaves were incubated in MES-KCl buffer (10 mM MES, 5 mM KCl, 50 μM CaCl$_2$, pH 6.15) under light for 3 hours with adaxial surface facing up. Once the stomata were fully open, the leaf epidermis was peeled from the adaxial surface. The epidermal peels were incubated in the buffer alone, the buffer with 10 μM ABA, or the buffer with 5 μM ABA. The stomata were observed under a microscope at 0, 15, 30 or 45 min after incubating in the solutions. The epidermal peel was observed under a compound light microscope to determine the length and width of stomata using a calibrated micrometer on the ocular lens. One ocular unit was equivalent to 0.4 μm.

Abscisic acid quantification

Abscisic acid quantification was done on leaf samples of plants after 14 days or 28 days of treatment. The samples were freeze dried, and abscisic acid measurement was done with a mass spectrometry by a collaborator at the University of Florida.

Statistical analysis

Statistical analysis was performed using R (programming language). Analysis of variance test was done with completely randomized design. Fisher's least significant difference (LSD) test (at 0.05 level of significance) was done to determine significant difference between means.

RESULTS

In a previous study, our lab identified RS, an alfalfa collection that is naturalized to the Grand River National Grassland in South Dakota (SD), with a greater WUE compared to the other ten alfalfa collections. To understand what contributes to a higher WUE in RS, we examined morphological and physiological parameters that may be associated with a higher WUE by comparing with two controls, AF and FR. AF is a commercial variety with a low WUE, and FR is another alfalfa collection adapted to South Dakota dry environment but with a lower WUE compared to RS (Anower et al. 2015)

Under the same conditions that were described in our previous study (Anower et al. 2015), we demonstrated again that RS showed the highest WUE compared to AF and FR (Fig. 2). Under well-watered condition, AF produced significantly more biomass than RS or FR. All three genotypes showed a significant reduction in biomass production after they were subjected to a progressive drought stress treatment for 28 days compared to well-watered

plants. The decrease in biomass production was 40%, 25%, and 31% for AF, RS and FR, respectively. All three genotypes produced similar dry shoot biomass under drought conditions (Fig. 2a). AF used the greatest amount of water under well-watered condition. RS and FR used a similar amount of water which was less compared to AF. Under drought stress conditions (W50 or 50% water regime), AF transpired more water than RS (Fig. 2b). As a consequence, WUE was similar in all three genotype in well-watered conditions, and it was increased in all three genotypes under drought conditions. The increase was 16%, 46%, and 27% for AF, RS and FR respectively. RS showed significantly higher WUE than AF and FR (Fig. 2c) as reported in our previous study.

Transpirational water use is closely associated with stomatal conductance. We thus examined the stomatal conductance on both sides of the leaf. Stomatal conductance of the adaxial surface of the leaf was similar among AF, RS, and FR under well-watered conditions. Stomatal conductance of the abaxial surface was the same when compared to that of the adaxial surface in AF. In RS and FR, however, stomatal conductance on the abaxial surface was significantly lower than that of the adaxial surface (Fig. 3). As a consequence, stomatal conductance of the abaxial surface in AF was significantly greater than those in RS and FR.

Water stress treatment resulted in a significant reduction in stomatal conductance of both sides of the leaf in all three genotypes. For the adaxial surface, stomatal conductance reduced 41%, 65% and 51% for AF, RS, and FR, respectively. For the abaxial surface, stomatal conductance reduced 65%, 72%, 68% for AF, RS, and FR respectively. As a consequence, RS showed the lowest stomatal conductance on both leaf surfaces, followed

by FR. In all three genotypes, stomatal conductance was lower on the abaxial surface than on the adaxial surface under water stress conditions (Fig. 3).

The difference in stomatal conductance in three genotypes may be due to a difference in stomatal density, thus stomatal number per unit leaf area was examined. Three genotypes had the same stomatal density on the adaxial surface. Stomatal density on the adaxial surface was greater compared to the abaxial surface under well-watered conditions in RS and FR, while AF has a similar number of stomata on both surfaces. Water stress treatment had no effect on stomatal density in AF, but slightly increased the stomatal density in RS (12%) and FR (11%) on adaxial surfaced and RS (8%) and FR (6%) on abaxial surface (Fig. 4).

An increase in stomatal density in RS and FR was unexpected, since they showed lower stomatal conductance. Thus, the stomatal pore area in three genotypes was investigated. An example of leaf stomatal aperture measuremnt was shown in Figure 5 and the results were summarised in Figure 6. The stomatal pore area on adaxial surface in RS and FR is the same but is significantly smaller than that in AF under well-watered conditions. Water stress treatment reduced stomatal pore size by 25%, 25%, and 17% in AF, RS and FR, respectively. RS showed the smallest stomatal pore area compared to AF and FR under water stress conditions (Fig. 6).

The data in Figure 5 represent the pore area of stomata on the adaxial surface after placing leaf on a stoma opening buffer (MES-KCl) for 3 hours under light. The data suggested that either plants under drought develop smaller stomata or some factors in the water-stressed leaf limited the stomatal opening to full size.

Since plants accumulate ABA under drought stress, and ABA is a major regulator to close stomata, we determined the amount of ABA in these plants and stomatal sensitivity to ABA treatment. We found that AF contained more ABA than RS under both well-watered and drought conditions. AF has 11 times and 1.7 times more ABA in well-watered condition at 14 days and 28 days of the experiment compared to RS. Under drought conditions, AF has 3 times more ABA at 14 days and 2.4 times more at 28 days. Both RS and AF showed an increase in ABA 28 days after drought. The increase was 60% for AF and almost 100% in RS (Fig. 7). Due to a high data variation, the increase was not statistically significant in RS. Even so, the amount of ABA accumulated in the leaf could not explain the small stomata aperture in RS, suggesting that RS may show a higher sensitivity to ABA compared to that in AF. We thus examined stomata response to ABA treatment.

Stomata of all three genotypes closed rapidly when the leaf epidermal peels were treated with 10 μM ABA. Although all three genotypes decreased the width of stomata significantly within the first 15 minutes of ABA treatment, the closure of RS was greater than AF and FR. The decrease in width was 14%, 20% and 17% for AF, RS and FR respectively. The slope of the line graph for first 15 minutes was -0.043, -0.059, -0.051 for AF, RS and FR respectively. RS also showed significantly smaller stomatal width at 30 and 45 min after ABA treatment. FR showed a significantly smaller stomatal width than AF but greater than RS 30 min after ABA treatment. FR showed the same stomatal width as AF at 45 min after ABA treatment. The width of stomata was similar among genotypes without ABA treatment and remained unchanged during measurement (Fig. 8).

We also examined the stomatal response to ABA in drought stressed leaves. The stomatal width was smaller compared to the well-watered plants (compare to Fig. 8) and stayed the

same size in an incubation buffer without ABA treatment. Treatment of 10 µM ABA also resulted in a rapid closure of stomata in all three genotypes. The decrease in width after 15 minutes was 15%, 24% and 16% for AF, RS and FR respectively (Fig. 9). The slope of the line was -0.042, -0.064, -0.044 for AF, RS and FR, respectively. RS showed consistently smaller stomatal width after ABA treatment compared to AF and FR. FR showed smaller stomatal width at 30 and 45min after ABA treatment compared to AF. After 15 minutes, the response to ABA of all three genotypes was similar (the lines are nearly parallel).

Since 10 µM ABA treatment caused a rapid stomatal closure in all genotypes, which may have made the difference less distinguishable at the early stages of ABA treatment, we thus measured the stomatal width after 5 µM ABA treatment. Again, ABA treatment resulted in closure of stomata but the rate of closure was smaller (compared to Fig. 8). In well-watered plants RS again showed the greatest reduction in stomatal width (16%), while FR showed the second greatest reduction (11%) and AF the least reduction (8%) (Fig. 10). The slope of the line was -0.024, -0.045, -0.030 for AF, RS and FR, respectively. The rate of stomatal closure was again similar after the first 15 minutes.

In water stressed plants, RS showed a rapid reduction in stomatal width within 15 min of ABA treatment. However, the rate of closure was similar among there genotypes after 15 minutes. FR and AF showed nearly identical response to 5 µM ABA treatment, except at 45 min where FR showed a significant reduction in stomatal width. The decrease in width of stomata after 15 minute was 8%, 19% and 11% for AF, RS and FR respectively (Fig. 11). The slope of line was -0.025, -0.052, -0.031 for AF, RS and FR respectively.

Leaf hairs increase the boundary layer and thus decreases water loss. We examined whether leaf hair features differ in the three genotypes. As shown in Figure 12a, RS showed the

densest leaf hair among three genotypes in well-watered conditions, it showed 36% and 12% more dense compared to FR and AF, respectively. Under drought conditions RS showed an 8% increase in leaf hair density, resulting in 43% and 16% more leaf hairs compared to FR and AF, respectively. Since drought stress frequently inhibits cell expansion in leaf and stem, an increase in leaf hair number in RS may simply be due to smaller cells and thus a greater cell density. Figure 13 showed that the epidermal cell density on abaxial surface is similar among three genotypes under well-watered condition. Thus, the greater leaf hair density in RS under well-watered condition was not due to smaller leaf cells. Under drought conditions, RS showed a 22% increase in cell density compared to well-watered plants. Thus, the results suggested that a decrease in leaf hair development occurred in RS under drought despite a greater density of leaf hairs when compared to well-watered plants. The same is true for FR plants (Fig. 12a and Fig. 13). We also examined the number of epidermal cells on adaxial leaf surface. In well-watered condition, AF, RS and FR has similar epidermal cell number. Under drought conditions, cell density increased in all three genotypes. The increase of cell number per mm^2 was 8%, 10% and 15% for AF, RS and FR, respectively (Fig. 13).

We also determined the length of leaf hairs. In well-watered conditions, RS showed 10% longer leaf hair than AF and was similar to FR. In drought stressed conditions, leaf hair length decreased 7% and 10% in RS and FR, respectively. AF, however, did not show a significant decrease in the leaf hair length after drought treatment. As a result, hair length was similar in RS and AF in drought stressed condition (Fig. 14).
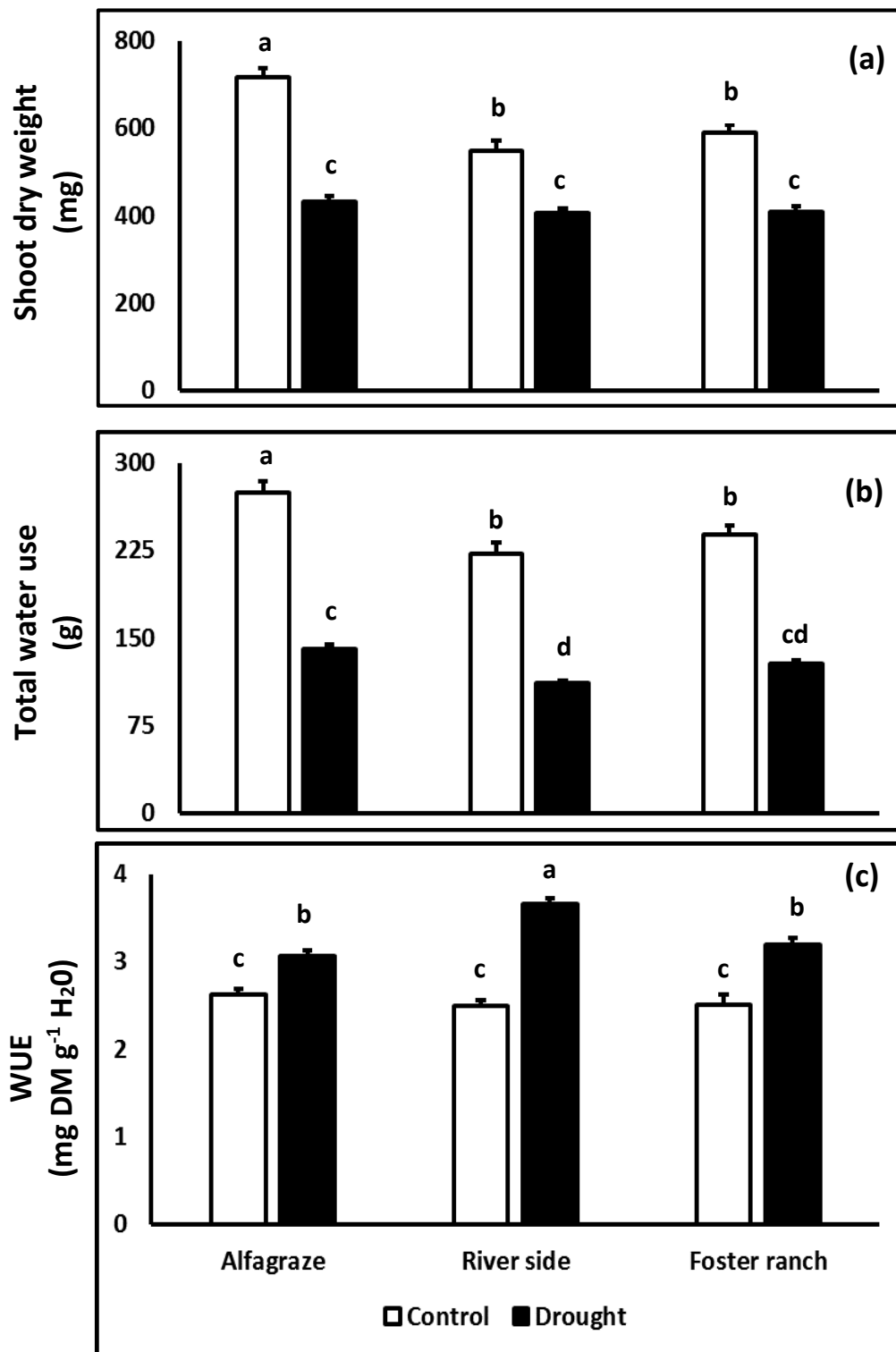
Figure 2. Net shoot dry matter (DM)(a), total water usage(b), and water use efficiency (WUE)(c) under well-watered and drought conditions in three different alfalfa collections. Different letters indicate significant difference (p<0.05). Data are shown as mean ± s.e. (n=21)
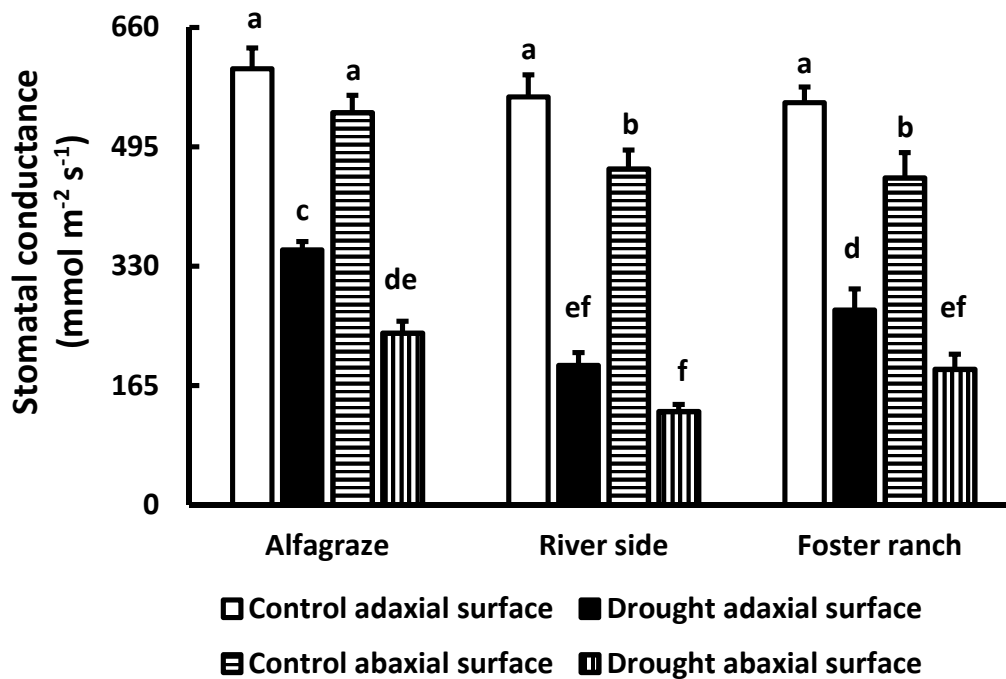
Figure 3. Stomatal conductance on adaxial and abaxial leaf surface under well-watered and drought condition. Different letters indicate significant difference (p<0.05). Data are shown as mean ± s.e (n=6)
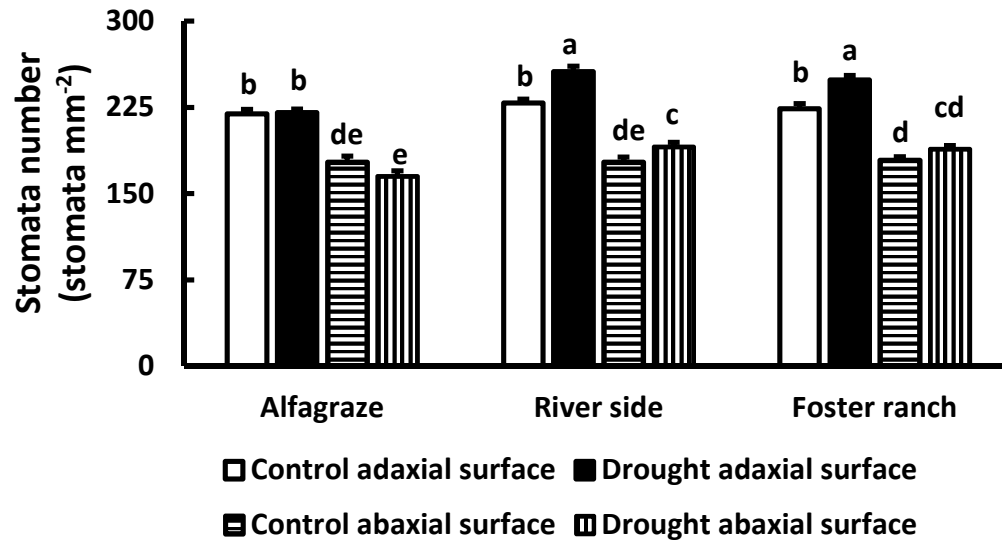
Figure 4. Stomata Density on adaxial and abaxial leaf surface under well-watered and drought conditions. Different letter indicates significant differences (p<0.05). Data are shown as mean ± s.e (n=35)

Figure 5. Measuring stomata width with calibrated ocular micrometer
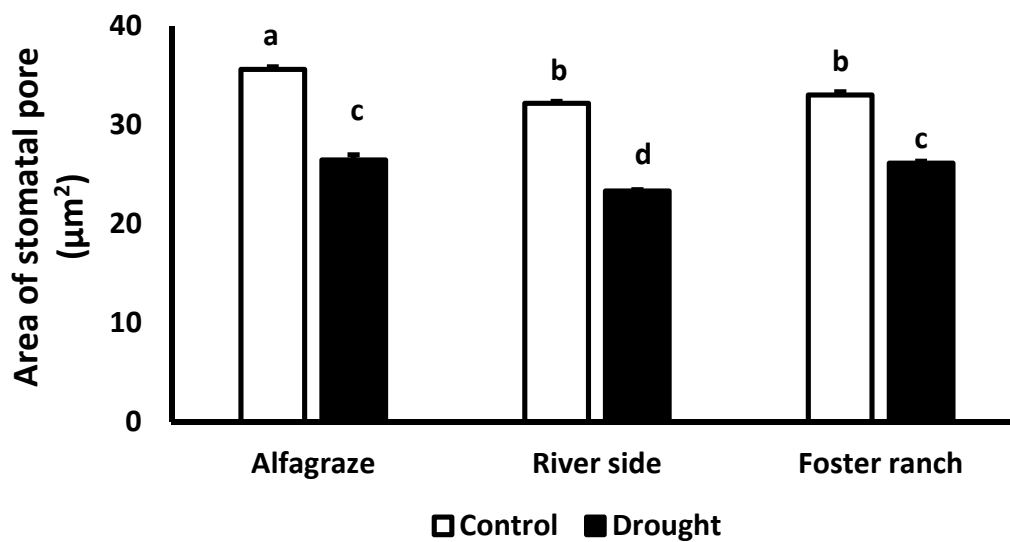
Figure 6. Area of stomatal pore opening of well-watered and drought stressed plants. Different letters indicate significant difference (p<0.05) Data are presented as mean ± s.e (n=90)
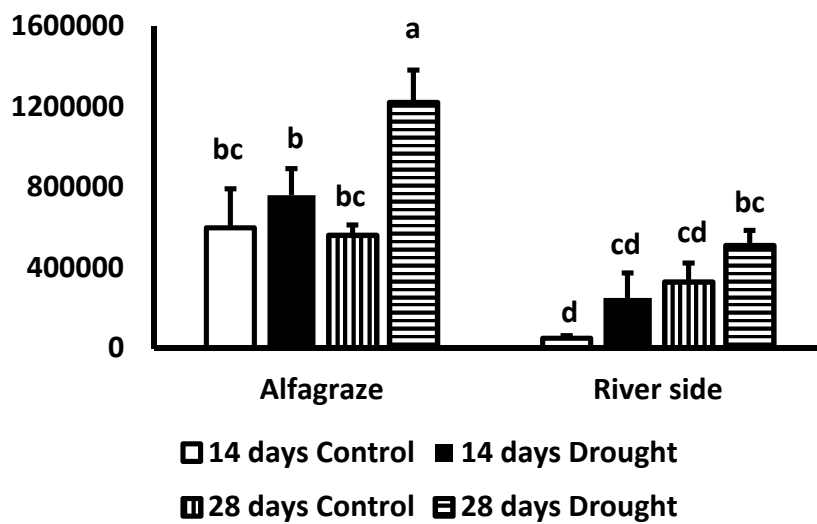
Figure 7. Abscisic acid accumulation in well-watered and drought stressed conditions. Different letters indicate significant differences. Data are presented as mean±s.e (n=3)
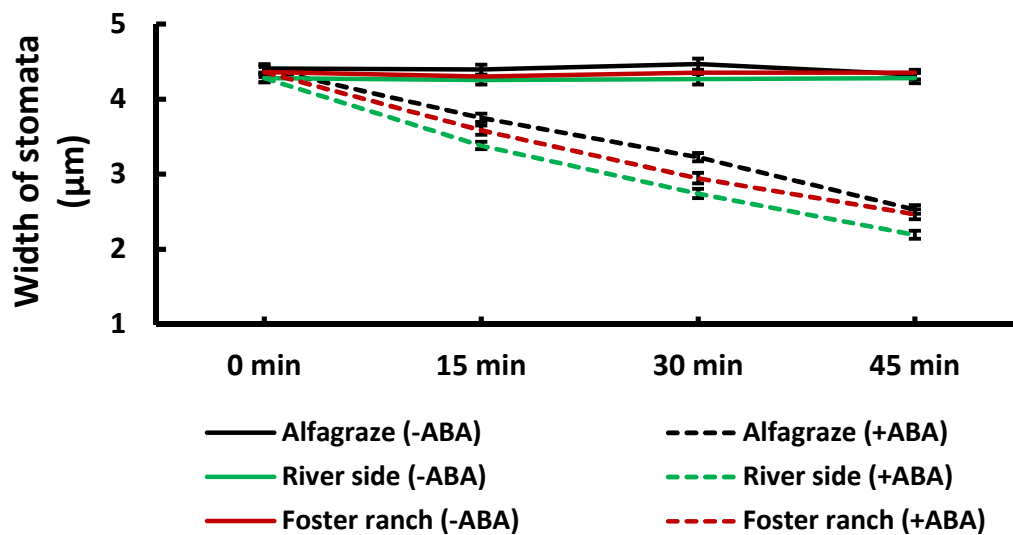
Figure 8. Stomatal closure of well-watered plants in response to 10 μM ABA treatment. Data are presented as mean ± s.e. (n=90)
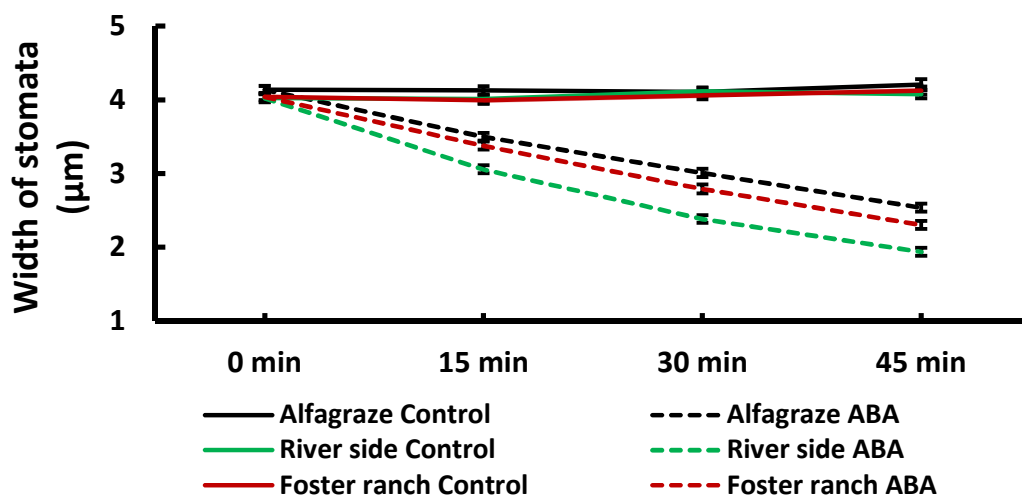
Figure 9. Stomatal closure of drought stressed plants in response to10 µM ABA treatment. Data are presented as mean ± s.e. (n=90)
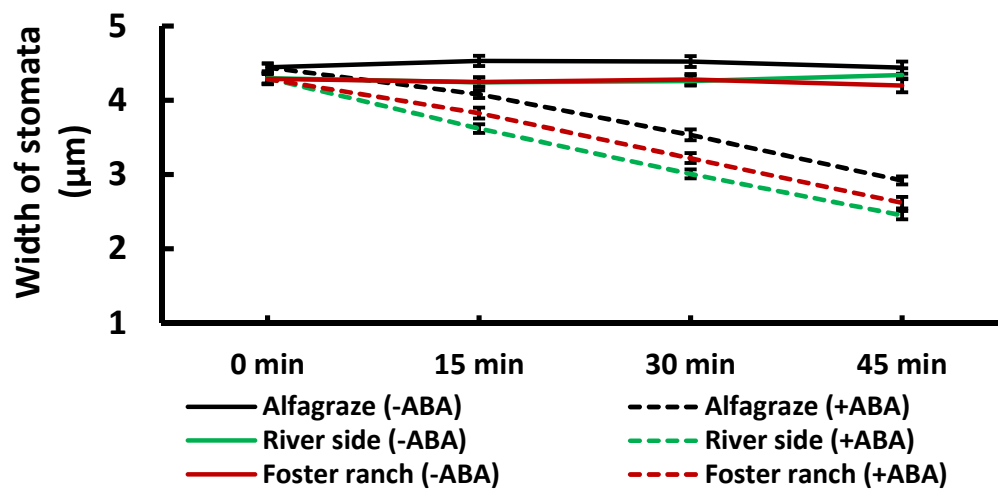
Figure 10. Stomatal closure of well-watered plants treated with 5 μM ABA. Data are presented as mean ± s.e. (n=90)
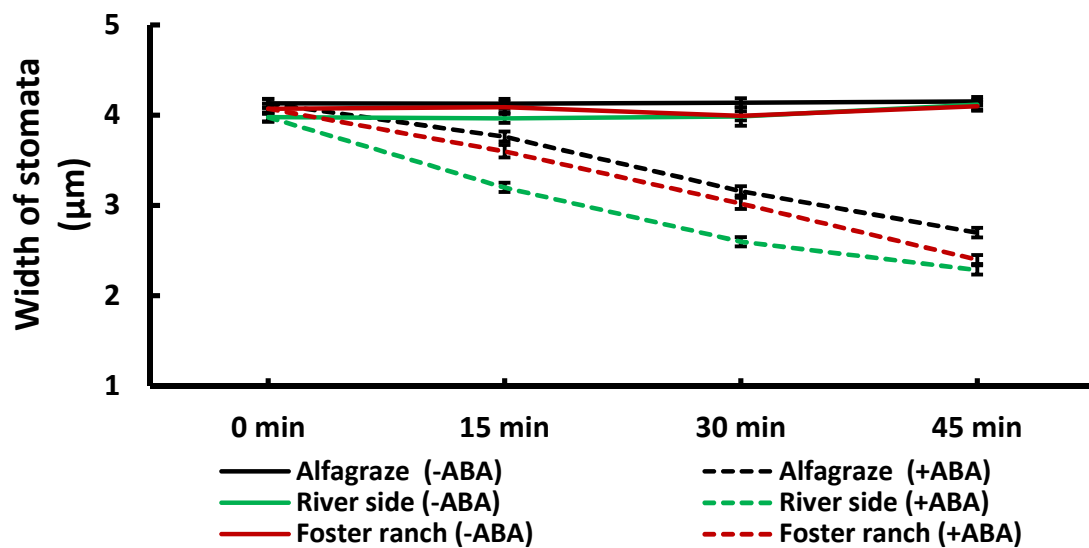
Figure 11. Stomatal closure of drought stressed plants treated with 5 μM ABA. Data are presented as mean ± s.e. (n=90)
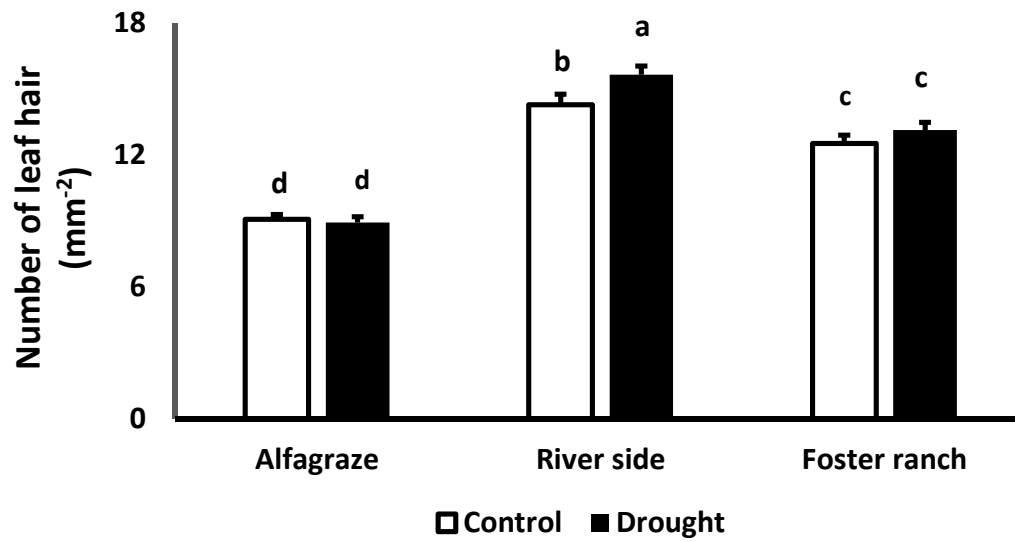
Figure 12a. Leaf hair number of well-watered and drought stressed plants observed on abaxial leaf surface. Different letters indicate significant difference (p<0.05). Data are presented as mean ±s.e (n=36)
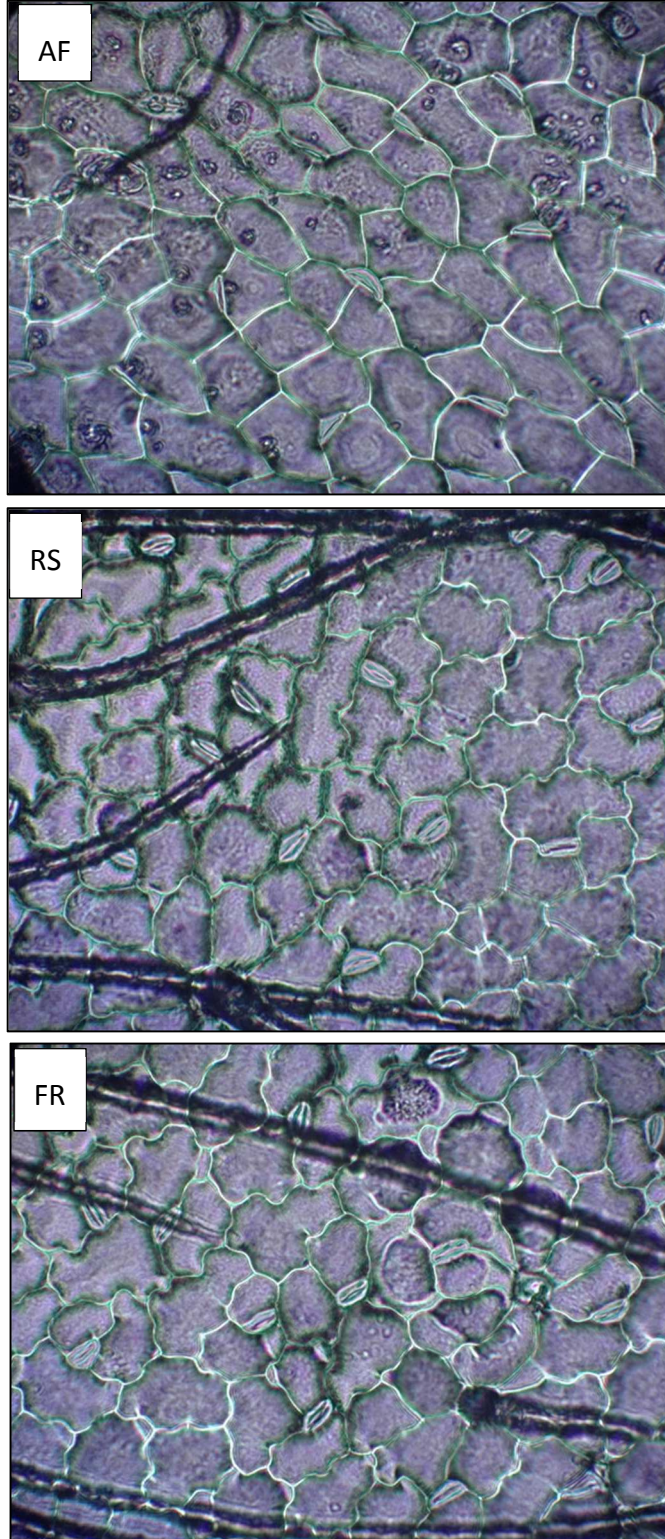
Figure 12b. Cell size in abaxial surface of AF, RS and FR
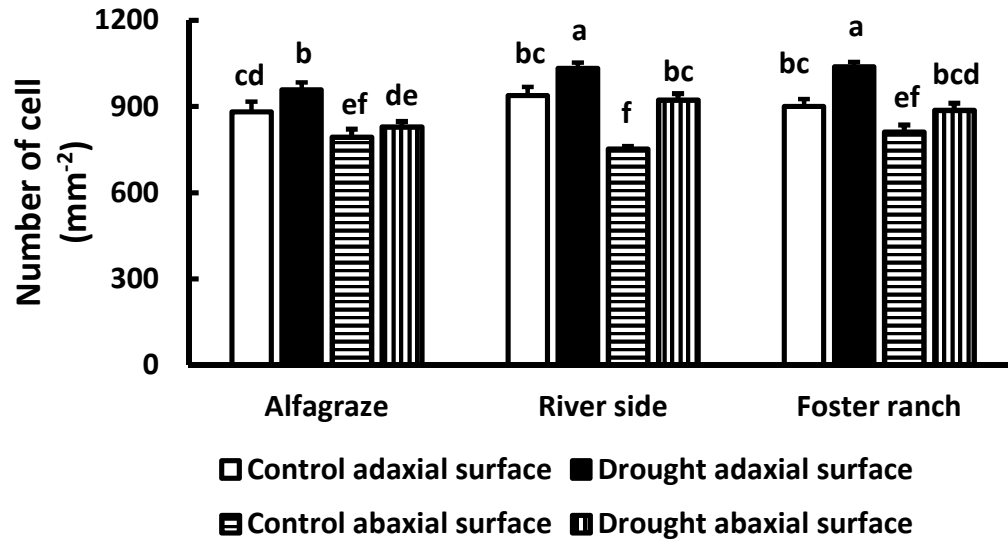
Figure 13. Cell number on adaxial and abaxial leaf surface under well-watered and drought condition. Different letters indicate significant difference (p<0.05). Data are shown as mean ± s.e (n=6)
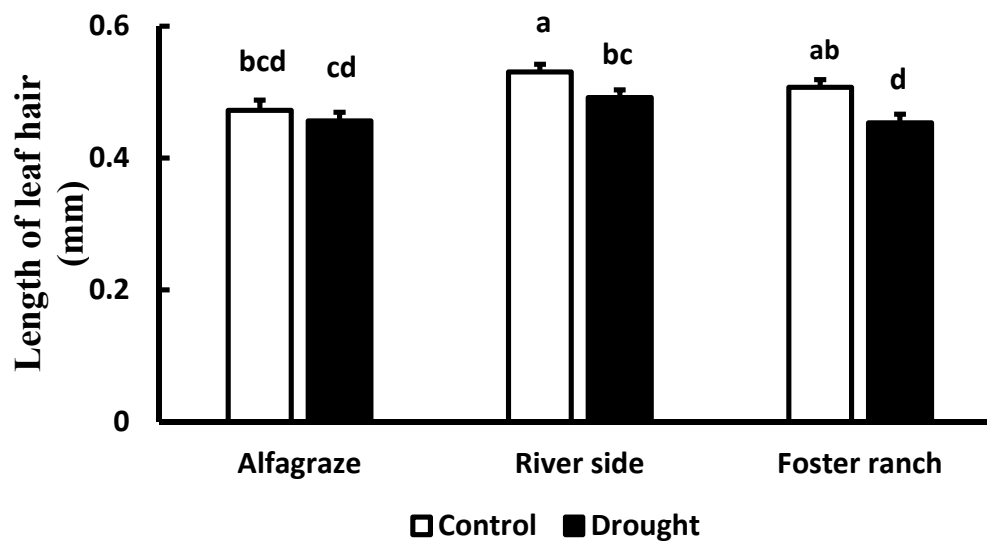
Figure 14. Leaf hair length of well-watered and drought stressed plants observed on abaxial leaf surface. Different letters indicate significant difference (p<0.05). Data are presented as mean ±s.e (n=60)

DISCUSSION

In an effort to improve WUE in alfalfa, our lab identified an alfalfa collection, RS, with a greater WUE among 11 alfalfa collections examined (ref). In this study, we investigated various traits that may contribute to a higher WUE in RS. AF performed the best in terms of biomass production under well-watered condition, but it consumed the greatest amount of irrigation water, resulting in a similar WUE to RS. The greater usage of water in AF is mostly due to a higher transpirational rate shown in Figure 2. The results also suggest that three alfalfa collections examined in this study may have similar activity in photosynthetic assimilation, since WUE is mostly decided by transpirational water loss and the capacity of photosynthetic assimilation (Keenan et al. 2013)

Under drought conditions, all three genotypes showed a significant increase in WUE. An increase in WUE during drought, especially under mild drought, is often due to the fact that inhibition of transpiration is more than inhibition of photosynthesis (Xu et al. 2010). This is probably true for our study, since the increase in WUE in all three genotypes is associated with a significant decrease in stomatal conducatnce and RS showed the the greatest reduction in stomatal conductance. Various studies have shown that the genetic variation in WUE is mainly due to variation in stomatal conductance but not net assimilation (Blum 2009; Yoo et al. 2009). A decrease in CO_2 assimilation under drought is moslty due to a redcution in stomatal conductance because a reduced condcutance also decreases influx of CO_2 for photosythesis. For this reason, we mostly investigated the features that may control stomatal conductance, including stomata density and size (Bergmann and Sack 2007; Xu and Zhou 2008), leaf hair (Picotte et al. 2007), stomatal

aperture size and stomatal movements (opening and closing) (Hetherington and Woodward 2003; Yoo et al. 2009).

With response to drought one might expect plants to decrease stomata number or density to minimize transpiration. Indeed, a decrease in stomatal density was correlated with a decrease in transpiration (Lake and Woodward 2008). Unexpectedly, we observed a significant increase in stomatal density in RS on both sides of the leaf, especially on the adaxial surface under drought. Thus, a change in stomatal density dose not contribute to the decrease in stomatal conductance in RS. Researchers has reported both decrease (Xu and Zhou 2008) and increase (Fraser et al. 2009; Yang and Wang 2000; Zhang et al. 2005; Ennajeh et al. 2010) of stomata number under drought conditions. An increase in stomatal density in drought condition has been proposed to increase transpiration in an attempt to increase nutrient uptake (Yoo et al. 2009; Hepworth et al. 2015). Since RS did not show an increase in stomatal conductance, it is unclear of the biological significance of an increase in stomatal density. Alfalfa is amphistomatous with more stomata on adaxial surface while most amphistomatous plants has more stomata on abaxial surface (Tari 2003; Willmer and Fricker 1996). While this presents a challenge to plants since this will enhance transpirational water loss and decrease WUE, having more stomata on the adaxial surface may be needed for water uptake and extraction for plants like alfalfa with a very deep root system. Thus, it is possible that an enhanced stomata density in RS and FR under drought may facilitate water uptake. Through examining leaf epidermal cell size and density, we found that the increase in stomatal density in RS and FR under drought could be explained by the greater cell density (due to reduced cell size in these two collections). In fact, RS

showed a greater increase in cell density than the stomatal density, suggesting that drought stress may inhibit the production of stomata in RS.

Since the change in stomatal density cannot explain the decrease in stomatal conductance in RS, we measured the size of stomatal pore. Stomatal pore size controls transpiration, CO_2 uptake and thus WUE (Bergmann and Sack 2007; Kim et al. 2010). We measured the stomatal pore area of the stomata on the adaxial leaf surface by estimating their maximum potential of opening after floating leaf epidermal peels in a buffer. Two reasons that we only examined the stomata on the adaxial surface are: 1) stomata on the adaxial showed more transpiration and 2) leaf epidermal were difficult to peel on abaxial surface due to leaf hairs. RS and FR showed smaller stomata apertures than AF, however, stomatal conductance was similar, suggesting stomata aperture was not directly correlated with stomatal conductance under well-watered conditions for these alfalfa plants. Nevertheless, drought stress decreased stomata aperture in all three genotypes, which is correlated with a decrease in stomatal conductance in all the genotypes. In addition, RS showed the smallest stomatal aperture and showed the smallest conductance. A decrease in stomatal pore size during drought was reported by (Bosabalidis and Kofidis 2002). It is reported that size and density are negatively correlated and a smaller stomatal size and a higher density is associated with high WUE in *Arabidopsis* (Franks et al. 2009). Thus, RS may have employ the same strategy to improve its WUE under drought.

Another important leaf trait that can affect transpiration is leaf hairs, also known as trichomes. They play various roles like structural defense against herbivory, fungal infection and against abiotic stress like drought, heat and excess light and uv radiation (Hauser 2014). They form a boundary layer on leaf surface and resist transpiration. RS

showed a significantly higher number of leaf hairs compared to AF and FR under both well-watered and drought conditions. In addition, RS is the only genotype showed an increase in leaf hairs. An increase in leaf hairs under drought has been reported and it is linked with a decrease in transpiration (Wuenscher 1970) and increase WUE (Picotte et al. 2007; Choinski and Wise 1999). Since RS also showed an increase of epidermal cell density on the abaxial surface under drought, the increase in leaf hair density is probably due to the change in cell density. While RS showed an increase in leaf hair density under drought, RS and FR also showed a significant reduction in hair length. It is not clear how this will affect stomatal conductance. Since RS and AF has the same length of leaf hairs and RS showed a lower stomatal conductance despite a greater stomatal density, it is very possible that the leaf hair density may play a more important role in impacting stomatal conductance.

It is very interesting that alfalfa has more stomata and higher conductance on the adaxial surface, while leaf hairs are only observed on the abaxial surface. Thus, two sides of the leaf may use different mechanisms for regulating transpiration, i.e. regulation of stomatal aperture for the stomata on the adaxial surface may be the key to regulate stomatal conductance.

ABA is accumulated in plants under drought and plays a major role in closing stomata and reducing transpiration, resulting in a higher WUE in plants (Guo et al. 2016; Kim et al. 2010). We found that the ABA content after 14 days of drought treatment increased slightly in both AF and RS compared to well-watered plants but the increase was not statistically significant, indicating that the intensity of drought stress was minimum at 14 days. AF plants, however, showed a significant increase in ABA content after 28 days of drought

treatment, while RS did not show a significant increase. The results were surprising since the plants were subjected to a similar stress level adjusted based on their transpirational rate and a reduction in biomass production and stomatal conductance in RS was obvious. No changes in ABA content in RS suggests that an ABA accumulation may not be a major mechanism in RS to control stomatal conductance under drought. Therefore, the amount of ABA accumulated in stressed plants could not explain a higher WUE and smaller stomatal aperture in RS. It is noticeable that there is an increase in ABA content in RS under well-watered conditions when compared the plants at 28 days to that at 14 days. The results suggested that an endogenous ABA level may also under a developmental control.

Since an ABA accumulation under drought did not seem to be involved in regulating WUE in RS, we examined response of stomata to ABA treatment to determine whether the sensitivity to ABA has changed. Although the width of stomata of all three genotypes was decreased in response to ABA, RS showed a greater response than AF and FR in both well-watered and water-stressed plants within the first 15 min of treatment of two different ABA concentrations. Thus, an early and efficient response of stomata to ABA might also contribute to higher WUE in RS.

Differences in plants in response to ABA and stomatal closure have been reported (Chen et al. 2013; Hopper et al. 2014; Pantin et al. 2013; Rossdeutsch et al. 2016). ABA is produced in the root and transported into the shoot via xylem and received in guard cells for stomatal closure (Mishra et al. 2006; Comstock 2002; Davies et al. 2005). ABA induced stomatal closure is achieved through other signal molecules such as H_2, NO, and H_2O_2 (Xie et al. 2014). Thus, the difference is ABA response in RS vs AF might be due to a higher number of ABA receptors or a greater accumulation of different secondary messengers in

the ABA signaling pathway like NO, H_2O_2 in RS. We only measured stomatal sensitivity to ABA on the stomata of adaxial surface again due to the difficulty to make epidermis peels from the abaxial surface (due to the presence of leaf hairs). Differential regulation of adaxial and abaxial stomata for transpiration under drought (Pachepsky et al. 2000) and for light response (Yera et al. 1986; Turner 1970) has been reported. Abaxial stomata of broad bean were reported to be more sensitive to ABA than adaxial stomata and may use different pathways for ABA signal transduction on the abaxial and adaxial surface (Wang et al. 1998).

In summary, RS showed a higher WUE when compared to AF and FR under drought. This higher WUE in RS is associated with a reduced stomatal conductance. Through examining various morphological and physiological traits, we found that two sides of the leaf of RS may developed different mechanisms to reduce stomatal conductance. For the adaxial surface, an increased stomatal sensitivity to ABA may at least partially contribute to a low stomatal conductance and for the abaxial surface, an enhanced leaf hair density may at least partially, contribute to the lower conductance. RS is one of the alfalfa collections that have been naturalized to the Grand River National Grassland region where drought is frequent and severe. These complex morphological and physiological changes are a consequence of natural selection, allowing them to survive and reproduce under the arid growth conditions.

CONCLUSIONS

WUE is a complex trait given by the equation $WUE = \frac{biomass\ accumulation}{water\ use}$. Thus, WUE might be influenced by any trait that can increase or decrease biomass accumulation

(photosynthesis) and/or evapotranspiration. In our study, we revealed complex changes in morphology and physiology that may contribute to the regulation of stomatal conductance thus an improved WUE in RS under drought. RS developed smaller stomata under drought that might facilitate a more rapid stomatal closure when water is limited but enhance water and nutrient uptake when water is sufficient. RS has also exhibited different changes on two sides of the leaf that may contribute to the regulation of water loss. The abaxial surface developed a greater number of leaf hairs that can potentially increase the boundary layer resistance for transpiration. The adaxial surface developed the stomata with a greater sensitivity to ABA. Our study provided a great insight into the factors that may contribute to a high WUE in alfalfa. More studies are needed to identify other factors that may be important in controlling WUE in alfalfa. For example, both chemical and hydraulic signals are involved in stomatal regulation to minimize water use (Comstock 2002; Pantin et al. 2013; Tombesi et al. 2015). Thus, more investigation into root hydraulic conductance and root architecture is needed to understand their role in controlling WUE in alfalfa. It is also important to address the genetic and molecular mechanisms that determine the stomatal sensitivity to ABA. The knowledge developed in this study and in the future study will build a foundation for developing alfalfa with improved WUE.

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